ISOLATION OF CYTOKININ BIOSYNTHESIS AND METABOLIC GENES FROM WHITE CLOVER (*Trifolium repens* L)

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Abstract

The factors influencing senescence in white clover (*Trifolium repens* L.) are of considerable importance to the pastoral sector of New Zealand's economy. The plant hormones, ethylene and the cytokinins, have been implicated as having opposing influences on senescence. This project focused on the cytokinins. The rate limiting step in cytokinin biosynthesis is catalysed by isopentenyl transferase (IPT) and the primary enzyme in the degradation of cytokinins is cytokinin oxidase/dehydrogenase (CKX). Both *IPT* and *CKX* genes are present as multi-gene families. A reduction in the level of active cytokinins either via a decrease in *IPT* expression, or an increase in *CKX* expression, or both, would implicate the cytokinins in developmental leaf senescence in white clover.

White clover grows in a sequential pattern with leaves at all stages of development making it a good model for studying leaf development and senescence. A decrease in leaf chlorophyll is used as a marker for the onset of senescence. A micro-scale chlorophyll analysis was developed using the NanoDrop™ thus allowing tissue from the same leaflet to be used for gene expression and chlorophyll measurements. The pattern of chlorophyll changes was similar to that shown by Hunter *et al.*(1999) and Yoo *et al.*(2003) in white clover stolons used for ethylene research. Reverse transcriptase PCR (RT-PCR) and BLAST analysis was used to identify five putative *IPT* genes and seven putative *CKX* genes from white clover. RT-PCR demonstrated the expression of seven of these genes (*TrIPT1. TrIPT13, TrIPT15 TrCKX1, TrCKX2, TrCKX6*). Analysis with quantitative real-time PCR showed expression of *TrCKX2* increased markedly during leaf expansion and was consistently high during senescence, suggesting a potential role for CKX in facilitating the progression of senescence.

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
ACO	ACC oxidase
ACS	ACC synthase
ACT	Actin
bp	base pairs
cDNA	complementary deoxyribonucleic acid
°C	Degrees Celsius
CKX	cytokinin oxidase/dehydrogenase
cm	centimetre

Ct	Threshold cycle
c-t iso	*cis*-trans isomerase
cZ	*cis*-zeatin
d	days
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide-triphosphate
DZ	dihydrozeatin
g	gram
g	gravity
GAP	glyceraldehyde-3-phosphate dehydrogenase
h	hours
iP	N6-(D2-isopentenyl)adenine
IPT	adenosine phosphate isopentenyltransferases
M	Molar; moles per litre
mg	milligram
min	minute
ml	millilitre
mM	millimolar

ng	nanograms
nm	nanometre
P(SEE1)	senescence-enhanced maize promoter
PCR	polymerase chain reaction
PSAG12	promoter of SAG12
qPCR	Quantitative real-time polymerase chain reaction
RFU	Relative florescence units
RNase	ribonucleases
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
s	second
SAG12	Arabidopsis senescence associated gene
tRNA-IPT	tRNA isopentenyltransferases
tZ	trans-zeatin
µg	microgram
µl	microlitre

Chapter 1
Introduction

1.1 Background

Understanding the molecular mechanisms that regulate growth and development is one of the biggest challenges facing plant biologists. Cytokinins are a group of plant hormones that play an important role controlling plant growth and development and this project focuses specifically on their role in leaf senescence. The application of cytokinin to leaves, either exogenously (Richmond and Lang 1957) or through the ectopic expression of cytokinin synthase (Gan and Amasino, 1995; Rivero et al., 2007) delays senescence, but the molecular mechanisms regulating cytokinin levels during senescence have yet to be determined.

The rate limiting step in cytokinin biosynthesis is catalysed by adenosine phosphate isopentenyltransferases (IPT) (Miyawaki et al., 2004; Ye et al., 2006). IPT catalyses the synthesise of cytokinins from free adenine nucleotides via the transfer of an isopentenyl group to the N(6) of ATP and ADP (Kakimoto, 2001). *IPT* genes are present as a small multi-gene family, and differential expression of the family members allows for tissue specific regulation of cytokinin production (Miyawaki et al., 2004). The primary enzyme in the degradation of cytokinins is cytokinin oxidase/dehydrogenase (CKX) which catalyses the side chain cleavage of cytokinins (Schmülling et al., 2003; Ashikari et al., 2005). Cytokinin oxidase/dehydrogenase genes are also present as a multi-gene family, and differential expression of the family members allows for tissue specific regulation of cytokinin degradation (Werner et al., 2006).

White clover (*Trifolium repens L*) is an important agronomic plant and its stoloniferous growth pattern with sequential leaf development and senescence makes it a good model for studying gene expression during leaf senescence (Hunter et al., 1999). In this study white clover was used to investigate the expression patterns of *IPT* and *CKX* genes during leaf development and senescence.

1.2 White Clover

White clover is the most important legume species grown for sheep and cattle grazing in temperate regions worldwide (Laidlaw and Teuber, 2001). It is estimated that 15 M ha of clover are grown in Australasia, 5 M ha in the US (Marten et al., 1989) and 9.5 M ha in the Humid Pampa region of Argentina (Scheneiter et al., 2009). Caradus et al. (1996) estimate the value of white clover to the New Zealand economy to be in excess of $3 billion and of this, seed production accounted for $30 million annually. Harris (1998) estimated the value of white cover to dairy pastures as between $380 – $435/ha/year in the Waikato and Bay of Plenty. Recalculating the equations of Harris (1998) with today's values (dairy pay out of $5.50 kg/milk solids and urea price of $600 kg) the value of clover is between $580.59 - $657.67/ha/year.

White clover (*Trifolium repens L*) is an allotetraploid (2n = 4x = 32) formed by a hybridisation event between *T. occidentale* and *T. pallescens* (Ellison et al., 2006). *T. repens* grows as a sprawling branching stolon. The stolon consists of a series of nodes interspersed by internodes. Each node consists of two root buds, one trifoliate leaf and a lateral bud. New leaves are formed at the apical bud. Roots develop from the nodes when they are in contact with moist ground. White clover undergoes a process of developmental senescence. As stolon growth progresses, older leaves senesce followed by senescence of the basal end of the stolon. By this process a single branching stolon is divided into multiple individual clones. Plants trained across a dry surface as a single unbranched stolon display the full sequence of leaf development and senescence. Hunter et al (1999) used this growth pattern to develop a model for studying developmental leaf senescence in white clover.

1.3 Cytokinins

Kinetin was the first successfully purified and characterised cytokinin but was an artefact isolated from autoclaved herring sperm DNA (Miller et al., 1955; Miller et al., 1955; Miller et al., 1956). In their classic experiment Skoog and Miller (1957) demonstrated how shoot or root growth in tobacco tissue culture was dependent on the ratio between kinetin and auxin. In the same year Richmond and Lang (1957) showed that kinetin delayed senescence on detached *Xanthium* leaves. Over the last 50 years, cytokinins have been implicated in many aspects of plant growth and development, including apical dominance, root and shoot differentiation, vascular patterning, gravitropism, fertility, seed development, stress tolerance and senescence (Mok and Mok, 2001; Miyawaki et al., 2004; Riefler et al., 2006; Müller and Sheen, 2007).

1.3.1 Cytokinin synthesis

The first naturally occurring cytokinin to be isolated zeatin was isolated from maize (Letham, 1963). Naturally occurring cytokinins are N^6-substituted adenine derivatives. The naturally occurring cytokinins fall in to two groups; the isoprenoid cytokinins with an isoprene side chain and the aromatic cytokinins with a benzyl moiety attached to the side chain (Jameson and Brian, 2003). Within the isoprenoid cytokinins, the cytokinin-ribosides are formed by the removal of the phosphate groups from the cytokinin-ribotides (Mok and Mok, 1994). Free-base cytokinins are formed either by the removal ribose from cytokinins-ribotide (Mok and Mok, 1994) or by direct removal of the ribose 5'-monophosphate moiety from the cytokinin-riboside by LOG (Kurakawa et al., 2007)(Figure 1.1).

The four primary naturally occurring isoprenoid cytokinins, N^6-(D2-isopentenyl)adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ) and dihydrozeatin (DZ), are denoted by the structure of their prenyl side chain namely the presence, absence or stereoisomeric position of the hydroxyl group (Figure 1.1). As a general rule the free base forms of the cytokinins are the biologically active forms (Sakakibara, 2006). Bioassays and cytokinin receptor ligand binding studies have shown tZ to be the most active cytokinin, followed by iP, with cZ having little or no activity (Yamada et al., 2001; Nishimura et al., 2004; Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004; Romanov et al., 2006).

There are two different cytokinin biosynthetic pathways, the *de novo* pathway and the indirect tRNA pathway (Figure 1.1). The rate limiting step in both biosynthetic pathways is catalysed by isopentenyltransferases. There are two different groups of isopentenyltransferases, the adenosine phosphate isopentenyltransferases (IPT), which synthesise cytokinins from free adenine nucleotides via the de novo pathway (Kakimoto, 2001), and the tRNA isopentenyltransferase's (tRNA-IPT), which use specific tRNA species as their substrate (Golovko et al., 2002).

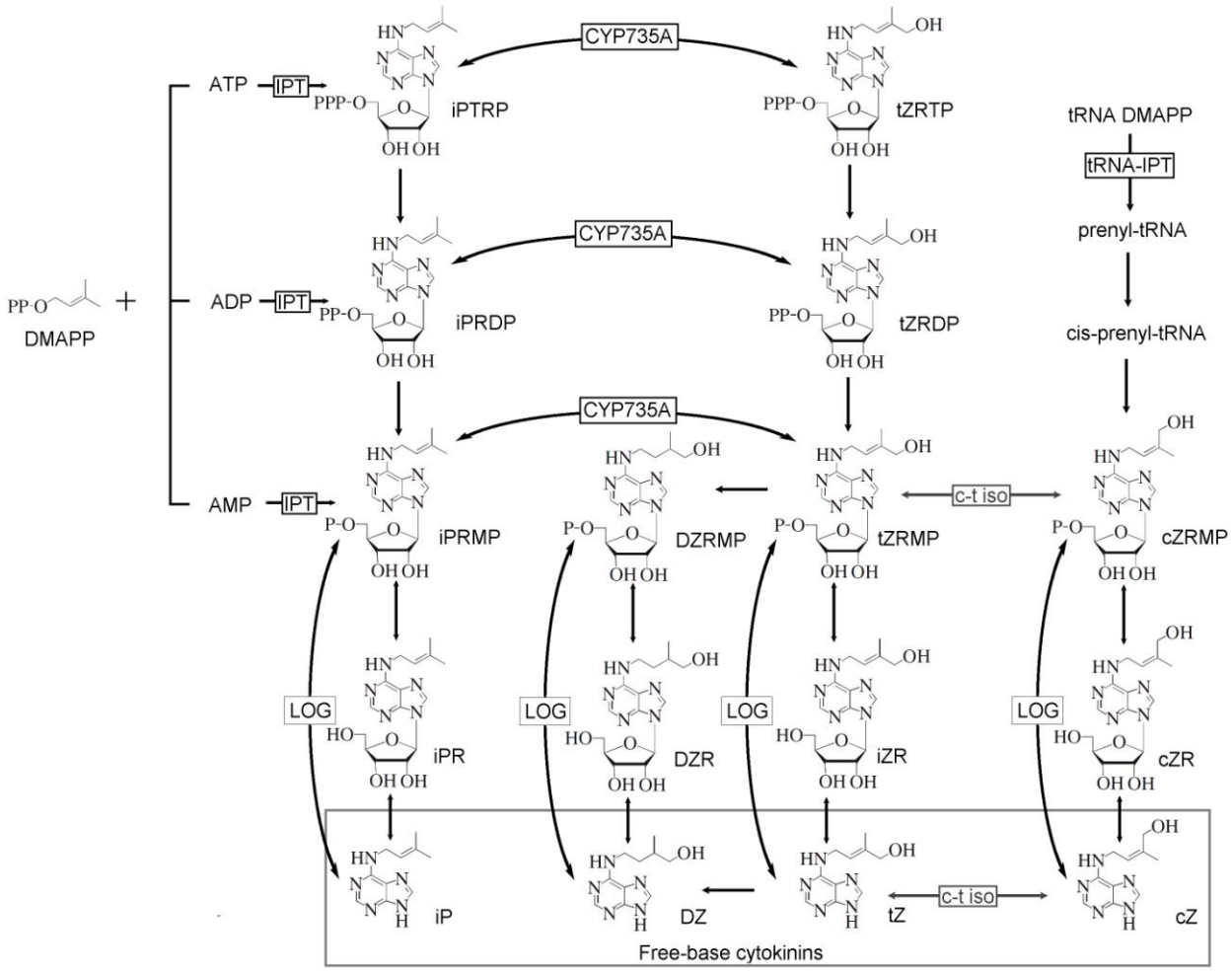


Figure 1.1, Current model of cytokinin biosynthesis:

Current model of cytokinin biosynthesis: cytokinins are synthesised from the free adenine nucleotides via the transfer of an isopentenyl group to the N^6-terminus of ATP, ADP or AMP by IPT. The *trans*-hydroxylation of the isopentenyladenine nucleotides to the *trans*-zeatin nucleotides is catalysed by CYP735A (Takei et al., 2004). LOG catalyses the direct synthesis of the freebase cytokinins N^6-(D2-isopentenyl)adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ) and dihydrozeatin (DZ) by the removal of the ribose 5'-monophosphate moiety from the cytokinin-ribotides to produce the freebase cytokinins (Kurakawa et al., 2007). *cis*-zeatin is synthesised from tRNA by tRNA IPT (Golovko et al., 2002). The *cis*-zeatin cytokinins may be converted to the *trans* forms by a *cis-trans* isomerase (c-t iso) (Bassil et al., 1993).

tRNA-IPTs catalyse the prenylation of the adenine adjacent to the 3' end of the anticodon at position 37 of some tRNA species using DMAPP as the isoprenoid precursor (Golovko et al., 2002). In plants the hydroxyl group of the prenylated tRNA is in the *cis* orientation and the resulting degradation of this produces *cis*-zeatin (cZ). *Cis*-zeatin is considered less biologically active than the other free base cytokinins (Yamada et al., 2001; Nishimura et al., 2004; Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004; Romanov et al., 2006). It is possible that cZ could be converted to tZ. A *cis-trans* isomerase has been identified (Bassil et al., 1993)(Figure 1.1) although any significant production of tZ has yet to be demonstrated (Veach et al., 2003; Quesnelle and Emery, 2007). Consequently, cZ is not thought to play a role in hormonal signalling. The de novo pathway is thought to be the major source of the biologically active cytokinins.

A cytokinin synthesis enzyme that operates independently of tRNA degradation was first isolated from the slime mould *Dictyostelium discoideum* (Taya et al., 1978). Taya et al. (1978) demonstrated a cytokinin synthesis pathway where 5'-AMP acted as the acceptor of the isopentenyl group. The first cloned *IPT* gene was isolated from the Ti plasmid of *Agrobacterium tumefaciens* (Akiyoshi et al., 1984; Barry et al., 1984). Despite considerable effort it was not until the *Arabidopsis* genome was sequenced that Takei et al. (2001) and Kakimoto (2001) independently found the first plant *IPT* genes.

Structurally and enzymatically, plant and microbial IPTs are quite different (Sugawara et al., 2008). Microbial and plant IPT enzymes differ in their preferred substrates. Microbial IPT utilises AMP whereas the plant IPTs prefer ATP and ADP (Kakimoto, 2001). The microbial IPT's *Tmr* and *Tzs* utilize both 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate diphosphate (HMBDP) and dimethylallyl diphosphate (DMAPP) as the isoprenoid donor, with HMBDP being the preferred substrate (Krall et al., 2002). Plant IPTs utilise only DMAPP (Kakimoto, 2001).

Plant *IPT* genes are present as multi gene families. For example, *Arabidopsis* has nine *IPT* genes of which two are *tRNA-IPTs* (Kakimoto, 2001; Takei et al., 2001), maize has at least eight *IPT* genes two of which are *tRNA-IPTs* (Brugiére et al., 2008) and rice has eight *IPT* genes (Sakamoto et al., 2006). The phylogenies of *IPT* genes show an early division of the *tRNA-IPTs* from the adenosine phosphate isopentenyltransferases and most of the *IPT* gene duplication events appear to have

occurred after the monocot dicot split (Kakimoto, 2001; Sakamoto et al., 2006; Brugiére et al., 2008). The individual *IPT* family members are expressed in a tissue specific manner, with expression predominately in the vasculature and actively growing tissues. In *Arabidopsis, IPT* family members exhibit specific expression patterns. For example, *AtIPT3* is expressed in the the leaf vasculature, *AtIPT5* in the root primordium, *AtIPT4* and *AtIPT8* in developing seeds and *AtIPT1* and *AtIPT7* floral tissues (Miyawaki et al., 2004; Takei et al., 2004). Of particular interest has been the identification of the pea gene *PsIPT1*, the expression of which is up regulated in stems following decapitation of the shoot apex (Tanaka et al., 2006).

1.3.2 Cytokinin degradation

In the current model of cytokinin metabolism, the cytokinins are degraded by the enzyme cytokinin oxidase/dehydrogenase (CKX) (Schmülling et al., 2003). CKX selectively degrades active cytokinins by the oxidative cleavage of the isoprenoid side chain (Galuszka et al., 2001; Schmülling et al., 2003) The first evidence of cytokinin oxidase activity was detected in a crude tobacco extract (Paces et al., 1971) and the first plant *CKX* gene was isolated from maize in 1999 by two groups independently (Houba-Hérin et al., 1999; Morris et al., 1999). Following this *CKX* genes have been isolated from wide a range of plants and are present as multi gene families (Schmülling et al., 2003). Seven CKX genes have been identified in *Arabidopsis* and eleven in rice (Werner et al., 2006). Transgenic plants over expressing CKX have lowered cytokinin levels and display the phenotypic effects of cytokinin deficiency such as increased root growth and retarded leaf and shoot growth (Werner et al., 2001). Gene expression studies in *Arabidopsis* (Werner et al., 2003; Werner et al., 2006) and maize (Brugiére et al., 2003) show differential expression of the family members. *CKX* genes are often expressed in small defined groups of cells, predominantly in the vascular tissues and areas of active growth (Brugiére et al., 2003; Werner et al., 2003; Werner et al., 2006) implying a role for CKX in fine tuning cytokinin levels in response to environmental and developmental cues in a tissue specific manner (Schmülling et al., 2003). One of the more interesting recent discoveries has been the identification of the gene responsible for an increased grain number phenotype in rice, as a null mutant for *OsCKX2* (Ashikari et al., 2005).

1.3.3 Glycosylation of cytokinin

The O-glycosylation of cytokinins offers an alternative route for the deactivation of cytokinins. Cytokinin O-glucosyltransferases have been identified in a wide range of plants (Pineda Rodo et al.,

2008). Cytokinin O-glucosides are not believed to be biologically active which is supported by the lack of affinity of the cytokinin receptors CRE1/AHK4 and AHK3 for cytokinin O-glucosides (Spíchal et al., 2004). Cytokinin O-glucosides can be converted back to active forms by β-glucosidase (Brzobohaty et al., 1993). Given the reversible nature of O-glucosylation, it has been proposed that cytokinin O-glucosides are a storage form (Jameson, 1994). The constitutive expression of the *Phaseolus lunatus* L. zeatin O-glucosyltransferase gene *ZOG1* in maize, produced plants displaying a cytokinin deficient phenotype with increased zeatin-O-glucoside levels (Pineda Rodo et al., 2008). However, leaf zeatin levels were found to have increased and senescence was delayed. This increase in leaf cytokinin levels was also observed in tobacco over expressing CKX (Mýtinová et al., 2006). This implies that reducing the active cytokinin pool in leaves can induce the localised over production of cytokinin which in turn distorts leaf development resulting in delayed senescence. Several studies have found an increase in cytokinin O-glucosides preceding the onset of senescence although this is not the case for all plants (Van Staden, 1996; Taverner et al., 1999). It is possible that O-glycosylation of cytokinins may initiate senescence in some plants.

1.4 Leaf senescence

Senescence is the final stage of leaf development and is a process of recycling, whereby all the cellular components are broken down and transported from the senescent tissues to elsewhere in the plant. Senescence is triggered by both external environmental factors such as shade and nutrient and drought stress as well as internal factors such as age, seed development and plant hormones (Lim et al., 2007). The mechanisms controlling senescence have evolved to optimise the survival and reproductive success of the plant. Although advantageous for the plant, this is not necessarily the optimal situation for the farmer. For example, drought-induced senescence may allow a plant to quickly complete its life cycle, but can dramatically reduce yield for the farmer (Rivero et al., 2007). Understanding the mechanisms that control senescence will allow the breeding of plants with higher nutritional value and increased tolerances to stresses such as drought.

1.4.1 The hormonal control of senescence

1.4.1.1 Ethylene and Senescence

Ethylene regulates numerous physiological processes, including promoting senescence (Gan, 2004). The first step in ethylene biosynthesis is catalysed by the enzyme ACC synthase (ACS) which converts S-adenosylmethionine to 1-aminocyclopropane-1-carboxylate (ACC) (Yang and Hoffman,

1984). This was believed to be the rate limiting step in ethylene biosynthesis. The second enzymatic step in ethylene biosynthesis is catalysed by ACC oxidase (ACO) which convertes ACC to ethylene (Yang and Hoffman, 1984) Attenuation of ethylene biosynthesis or action retards senescence (Clarke et al., 1994). In detached broccoli florets ACO expression increases with the onset of senescence (Gapper et al., 2005). Suppression of *ACO* expression in broccoli by expression of antisense *ACO* transcript delayed senescence in detached leaves and florets (Gapper et al., 2005).

ACC oxidase is present as a small multi gene family of four members in white clover (Hunter et al., 1999; Chen and McManus, 2006). ACO has been shown to have tissue specific and developmentally regulated expression and is proposed to fine tune ethylene production (Chen and McManus, 2006). ACO species were shown to be differentially expressed in clover leaves throughout development. *ACO1* was expressed primarily in the shoot apex, *ACO2* in expanding and mature leaves and *ACO3* and *ACO4* were expressed in senescing leaves (Hunter et al., 1999; Chen and McManus, 2006). The presence of senescence specific *ACO* genes in clover, implicate ACO in the molecular pathways regulating senescence.

1.4.1.2 Cytokinin and senescence

Senescence was one of the first developmental processes shown to be influenced by cytokinins. Richmond and Lang (1957) showed that kinetin delayed senescence in detached leaves. Cytokinin was subsequently shown to delay senescence in cuttings (Leopold and Kawase, 1964) and whole plants (Fletcher, 1969; Noodén et al., 1979). Furthermore a number of studies have shown a relationship between a reduction in endogenous cytokinin levels and the progression of senescence (Van Staden et al., 1988; Singh et al., 1992).

Following the discovery of the *Agrobacterium tumefaciens* isopentenyltransferase genes, a number of groups have expressed *IPT* in a range of plants (Smart et al., 1991; Li et al., 1992; Hewelt et al., 1994). The resulting transgenic plants had elevated levels of cytokinins and senescence was delayed. Aside from delayed senescence these plants displayed a number of other developmental abnormalities caused by the unregulated production of cytokinins such as reduced apical dominance, retarded root growth and reduced size (Smart et al., 1991; Li et al., 1992; Hewelt et al., 1994; Gan and Amasino, 1996).

Gan and Amasino (1995) identified an *Arabidopsis* senescence associated gene, *SAG12*, which is up

regulated at the onset of senescence, Gan and Amasino (1995) expressed *IPT* under the control of the promoter of *SAG12* (*PSAG12*). Expression of *IPT* by *PSAG12* has an auto regulatory affect such that senescence induces the expression of *IPT* which produces cytokinin that inhibited senescence and subsequently reduced *SAG* expression. Although *PASG12-IPT* transformed plants developed normally, these plants have altered source sink relationships (Jordi et al., 2000; McCabe et al., 2001; Cowan et al., 2005).

To investigate the classical model of roots as the source of the cytokinins controlling development, McKenzie *et al.*(1998) produced transgenic tobacco plants with *IPT* under the control of a root specific Cu inducible promoter. When grown in the presence of Cu these plants had elevated cytokinin levels in leaves and leaf senescence was delayed. These results were contrary to previous grafting studies (Faiss et al., 1997) and showed that the role of root sourced cytokinin in regulating development could not be ignored.

Robson et al. (2004) demonstrated a delay in senescence in maize by expressing *IPT* under the control of a senescence-enhanced maize promoter (P(SEE1)). In the most extreme phenotype, leaf yellowing was completely eliminated. Leaves went straight from green to bleaching. However, the inability to recycle nitrogen from old leaves resulted in chlorosis in emerging younger leaves. More recently, a drought stress specific inducible promoter, $P_{SARK,}$ was identified by Rivero et al. (2007). Rivero et al. (2007) transformed tobacco with a P_{SARK}*::IPT* construct that expressed *IPT* in response to drought stress. The resultant plants were resistant to drought-induced senescence but otherwise phenotypically normal.

Less work has been done regarding CKX and senescence. CKX over expressing tobacco (Werner et al., 2001) and *Arabidopsis* (Werner et al., 2003) plants did not show premature senescence as would be expected in a cytokinin deficient plant. Tobacco plants over expressing CKX had an altered phenotype and the observed delay in senescence may be due to an elongation of the developmental cycle (Mýtinová et al., 2006). This does not preclude CKX expression as a regulator of senescence as these plants had a large number of developmental abnormalities. Although cytokinin levels were reduced, this reduction may not have been sufficient to affect senescence in these abnormal plants.

1.4.1.3 Cytokinin and ethylene interactions

While cytokinin delays senescence, ethylene promotes senescence. It has been proposed that

cytokinin may inhibit post harvest senescence in broccoli by desensitizing the plant to ethylene (Clarke et al., 1994). Furthermore, Chang et al.(2003) found that ectopically expressed cytokinin in petunia delayed corolla senescence and reduced sensitivity to ethylene. Gapper et al.(2005) investigated the response of the ethylene biosynthesis genes, ACO and ACS, to cytokinin in senescing broccoli florets. They found application of the synthetic cytokinin 6-BAP delayed harvest-induced senescence while increasing the expression of ACS but down regulating ACO expression. Gapper *et al.*(2005) then proposed a model whereby cytokinin regulates post harvest senescence in broccoli by inhibiting either ethylene perception and/or biosynthesis.

The role of ethylene biosynthesis during developmental leaf senescence has been the topic of much research (Hunter et al., 1999; Gong and McManus, 2000; Yoo et al., 2003; Murray and McManus, 2005; Chen and McManus, 2006). Members of the ACS and ACO gene families have developmentally specific patterns of expression; of particular interest is the senescence specific expression of *Tr-ACO3* (Hunter et al., 1999). Given the interplay between ethylene and cytokinin during senescence, in this study we aimed to investigate biosynthesis and degradation of cytokinin during clover leaf senescence.

1.5 Hypothesis and Objectives

As increasing cytokinin has been shown to delay senescence, and stress induced senescence is correlated with a reduction in cytokinin levels it follows that reduction in cytokinin levels may be a senescence inducing factor.

This project is based on the hypothesis that cytokinin homeostasis plays a pivotal role in regulating leaf senescence and that tissue specific cytokinin levels are regulated through expression of the *IPT* and *CKX* genes. Therefore, the expression patterns of members of the *IPT* and *CKX* gene families might be expected to change at the onset of senescence with either a reduction in *IPT* expression, or an increase in *CKX* expression, or both.

The first objective of this study was to isolate the clover *IPT* and *CKX* genes and to develop a quantitative real-time PCR assay for these genes.

The second objective was to investigate whether the onset of senescence in white clover (*Trifolium repens* L) was associated with changes in the expression patterns of *IPT* and *CKX* gene family members in the leaves.

The third objective was to develop a micro-scale chlorophyll assay to enable both gene expression and chlorophyll analyses of individual clover leaflets, chlorophyll being a measure of leaf development and senescence (Yoo et al., 2003).

Chapter 2

General Materials and Methods

2.1 Plant Growth Conditions

2.1.1 Trifolium repens

Two sources of white clover plant material were used in this study;

- Free growing white clover (*Trifolium repens L.*) plants harvested from the lawns around the University of Canterbury (grid reference 43.523147S, 172.587297E), were used for preliminary gene isolation and assay development.

- *Trifolium repens* L. genotype 10F (AgResearch Grasslands, New Zealand) was the primary experimental cultivar used. Plants used were grown from cuttings kindly donated by Professor Michael McManus, Massey University, Palmerston North.

2.1.2 Cultivation of *Trifolium repens* L. genotype 10F

Stock plants were grown in the glass house under natural light. Cuttings from the stock plants were cultivated in potting mix with slow release fertiliser following the procedure of Hunter *et al.* (1999). Apical cuttings with four nodes were taken from stock plants. Leaves were excised at the petiole stolon junction leaving the first two apical leaves and planted in seed trays with the basal node buried in potting mix. Six cuttings were grown per tray. Cuttings were trained as a single stolon over dry white plastic, three in each direction (Figure 2.1). All lateral out growth was excised once a week. These plants were grown in the glass house under natural light conditions from 18 March to 22 April 2008.

Figure 2.1 Clover plants

Six clover plants trained as a single stolon over dry white plastic foil.

2.2 Sample collection and processing

All plant tissue samples were collected in 1.7 ml centrifuge tubes and frozen in liquid nitrogen and stored at -80°C. Leaves were excised from their petiole with Leaf 1 being designated as the first fully emerged apical leaf. The tip consisted of all the tissue up to the first leaf. Young stem tissue consisted of all the tissue between the apical tip and half way between the 2nd and 3rd node with the leaves and petioles excised. All above ground plant tissues were frozen as they were harvested. Working quickly, roots and nodules were washed free of soil, rinsed with distilled water and patted dry. The nodules were then excised from the roots with tweezers, and roots and nodules were frozen separately.

Two methods were used for preparing samples for RNA extraction: grinding and pulverizing. Leaves and apical tips were hand ground with a plastic pestle in 1.7 ml centrifuge tubes cooled with liquid nitrogen. This method was used for preparing leaf samples as it results in a fine homogeneous powder. This allowed chlorophyll and RNA extractions to be carried out on the same leaf.

Roots, stems and nodules were pulverized with the stainless steel tissue pulverizer (

Figure 2.2). Prior to use the pulverizer was cooled with liquid nitrogen. A sample of frozen tissue was placed in the pulverizer cylinder with a little liquid nitrogen. Once the nitrogen had boiled off the piston was inserted. With the unit on a solid surface the piston was struck with a hammer a few times. The resulting compressed tissue wafer was quickly broken up with a cooled spatula and transferred to a pre chilled 1.7 ml centrifuge tube and placed in liquid nitrogen. Care was taken throughout to avoid thawing the sample.

Figure 2.2 Pulverizer

The stainless steel tissue pulverizer: a frozen sample is placed in the cylinder and the piston inserted. The sample is shattered and compressed into a thin disk by striking the piston with a hammer.

2.3 Bioinformatic Methods

2.3.1 Gene identification and characterisation

Both the Public and Pastoral Genomics sequence database where screened for clover and legume *IPT* and *CKX* homologues using BLAST. Sequence data was aligned by the hypothetical peptide sequences to *Arabidopsis* and legume homologues in MEGA4 (Tamura et al., 2007), using a combination of the CLUSTALW alignment tool and by manual sequence alignment.

2.3.2 Phylogenetic analysis

The phylogenetic relationship between genes was determined by two means. BLAST was used to identify homologous genes from the sequence data bases, and phylogenetic trees were constructed in MEGA4 (Tamura et al., 2007) using the Neighbour Joining and Maximum Parsimony methods.

2.4 RNA isolation and cDNA synthesis

RNA was extracted from clover tissues using two different methods, TRIzol® / TRI Reagent® (Section2.4.1) or BP-10 spin column total RNA Miniprep (Section 2.4.2). The integrity and quality of isolated RNA was assessed by running 1 µl of the samples on a 1% (w/v) agarose gel (Section2.7). The concentration and purity of the RNA was assessed by spectrophotometry (Section 2.7.2)

RNA is very susceptible to degradation. The RNA degrading enzymes, ribonucleases (RNase) are prevalent throughout the environment and within plant tissues and the utmost care must be taken to prevent contamination of RNA samples. To prevent RNA degradation all tissue samples were immediately frozen in liquid nitrogen and stored at -80°C. Liquid nitrogen was used to prevent samples from defrosting during grinding or pulverising. To eliminate RNase from the work environment, work surfaces and pipettes were treated with RNase Zap (Invitrogen). Hydrogen peroxide (3%) was used to treat plastic and metal equipment. Glass and some metal equipment was baked at 300°C for 4 h. Commercially supplied RNase free pipette tips were used for all RNA work.

2.4.1 TRIzol® / Tri Reagent® RNA Extraction

TRIzol® and Tri reagent® RNA extractions were used to extract RNA from all plant tissues except

flowers. This extraction is a phenol-chloroform based extraction procedure. Phase separation of the aqueous phenol and organic chloroform containing fraction separates RNA into the aqueous fraction, leaving DNA on the interface and proteins in the organic fraction (Chomczynski and Sacchi, 1987).

A maximum of 100 mg of frozen and ground, or pulverised, tissue was added to 1ml of TRIzol® (Invitrogen) or Tri reagent® (Ambion) in a 1.7 ml centrifuge tube and incubated for 3 min at room temperature. The tubes were then centrifuged at 11000 rpm at 4°C for 5 min. The supernatant was transferred to a new tube, 100 µl of chloroform was added and the mixture shaken vigorously for 15 s, incubated at room temperature for 10 min and then centrifuged for 15 min at 11000 rpm at 4°C. The upper fraction was transferred to a new tube and left to stand for 5 min at room temperature. RNA was precipitated with isopropanol (500 ml), mixed well and incubated at room temperature for 10 min, then centrifuged at 11000 rpm, 4°C to pellet the RNA. The supernatant was discarded and the RNA pallet washed with 1 ml 75% (v/v) ethanol, centrifuged at 11000 rpm, 4°C for 5 min, the supernatant discarded and the wash step was repeated. The remaining ethanol was aspirated and the pellet air dried for 5 min. The RNA was resuspended in 50 µl of DEPC water (Section 6.2.8) with 1x RNA Secure (Invitrogen). The samples were then incubated at 65°C for 15 min to deactivate any RNase and stored at -20°C.

2.4.2 BP-10 Spin Column Total RNA Minipreps Super Kit

The BP-10 Spin Column Total RNA Minipreps Super Kit (Bio Pioneer) utilises a spin column with an imbedded membrane that selectively binds RNA. Nucleotides, proteins, salts and other impurities are spun from the sample via a series of washes. Pure water was then used to elute RNA from the membrane which was then spun into a collection tube.

Total RNA was extracted from samples of up to 100 mg of frozen and ground, or pulverised, tissue. Extractions were carried out according to the manufacturer's instructions with the following modifications: For extractions from flowers, RNA was eluted from the membrane in two steps, first 30 µl DEPC water was used to elute RNA following the standard protocol. Then 20 µl of the flow through was returned to the membrane followed by 20 µl of DEPC water, then spun back into the collection tube as for the first elution step.

Sufficient 25x RNA Secure (Invitrogen) was added to make a final concentration of 1x RNA Secure

and samples were incubated at 65°C for 15 min, then frozen at -20°C.

2.4.3 DNase treatment

Contaminating DNA was removed from RNA by treating RNA with DNase. DNase (2 µl), 10 × DNase (2 µl) and RNA (16 µl) were incubated at 37°C for 10 min to digest DNA. To degrade the DNase (2 µl), 25mM EDTA (1 µl) was added, the mixture was incubated at 65°C for 15 min, then frozen at -20°C.

2.4.4 cDNA synthesis

Extracted RNA was converted to cDNA by reverse transcription. Approximately 1 µg of RNA was mixed with 1 µl (100 pmole) of random primers (pDN6) and 1 µl (500 pmole) oligo (DT) primers and made up to 10 µl with DEPC treated water (Section 6.2.8). The solution was incubated at 65°C for 10 min and then placed on ice. RT master mix (10 µl) (Section 6.2.6) was added to the RNA primer mix and incubated initially at 42°C for 60 min, and then at 70°C for 15 min to deactivate the enzyme. The cDNA was diluted 5 fold with TE buffer (Section6.2.4) and stored at -20°C.

2.5 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was used to amplify DNA fragments. PCR reactions were performed using Taq PCR reagents from Roche (Section6.2.7). Reactions were carried out following the manufacturer's instructions. Where multiple samples were being used, a master mix without the template and/or primers was prepared.

The standard PCR program consisted of 35 cycles of 94°C for 40 s, 45-56°C for 40 s, 72°C for 40 s followed by one cycle of 72°C for 5 min then held at 4°C. When cDNA was being used, an initial cycle of 94°C for 5 min, 40°C for 5 min, 72°C for 5 min was performed to complete synthesis of the second DNA strand. Thermo-cycling was performed on either a Bio-Rad DNA Engine Peltier Thermal Cycler or a MJ Research PTC-200 Peltier Thermal Cycler.

2.6 PCR primer design

Primers for PCR were designed using Primer Premier™ 5.00. Care was taken designing primers to minimise potential primer dimers, false priming and hairpin structures. Primers were designed with lengths between 18 and 25 nucleotides and melting temperatures (Tm) between 55°C and 65°C.

2.7 DNA/RNA Quantification

Both agarose gel electrophoresis and spectrophotometry were used for quantifying the mass, integrity and purity of DNA and RNA samples. While spectrophotometry provides an accurate measurement of the concentration of DNA/RNA and the purity of the sample, it can not determine the integrity or determine the concentration of individual parts of mixed samples. Agarose gel electrophoresis was used to assess the integrity and concentration of the samples.

2.7.1 Agarose-gel electrophoresis

DNA/RNA agarose-gels were used throughout the project for quantifying and assessing the quality of RNA extractions and PCR products, and for separating and purifying DNA. Both 1% and 1.5% (w/v) agarose gels were used. The gels were made by mixing the appropriate weight of agarose in 25x TAE buffer (Abstract 6.2.1) and heating in the microwave until all the agarose had dissolved. Then 2 µl/30 ml of SYBR Safe™ DNA Gel Stain was added and swirled to mix. Once the solution had cooled to around 60°C it was poured into a cradle with the appropriate comb and allowed to set.

Gels were run between 60V and 120V for a sufficient time for the DNA/RNA to run 2/3 of the way down the gel. Samples were loaded with 6x agarose-gel loading dye (Appendix 6.2.5). A Bioline HyperLadder 1 (2 µl) (Appendix 6.3) was used for quantifying the size and weight of bands.

Gels were visualised using a Safe Imager™ blue-light transilluminator and/or a Chemi Genius2 BioImaging System (Syngene). Images were visualized, recorded and analysed using GeneSnap image acquisition software (Synoptics Ltd).

Integrity of RNA was assessed by the structure and distribution of bands (Figure 2.3). Integrity and weight of DA was determined by assessment of band structure and by comparison with the ladder.

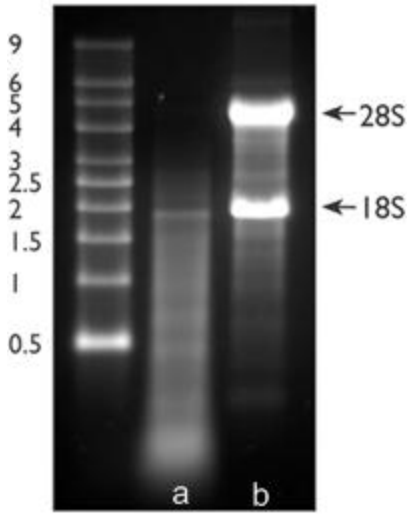


Figure 2.3 RNA integrity gel

RNA gel demonstrating, intact total RNA (b) and degraded RNA (a). Image is reproduced from Ambion's TechNotes 8(3), (www.ambion.com).

2.7.2 Spectrophotometry

A "NanoDrop™ " spectrophotometer (Thermo Fisher Scientific Inc.) was used to measure the quantity and purity of DNA/RNA in Samples. The NanoDrop utilises the absorbance peak of DNA/RNA (260 nm) to calculate concentrations and provides an estimate of purity by assessing the 260/280 nm and 260/230 nm ratios. Samples with, 260/280 ratios of between 1.8 – 2.0, and 260/230 ratios are generally accepted as pure (NanoDrop, 2005).

2.8 DNA Purification

For optimal sequencing and second round PCR reactions a pure DNA template is required. Gel purification and silica bead DNA purification were used to clean up PCR products.

2.8.1 Gel Purification

DNA was run in an agarose gel until bands had well separated. A Safe Imager™ blue-light transilluminator was used to visualise the DNA while bands were cut out. DNA was extracted from the gel using silica bead DNA purification.

2.8.2 Silica bead DNA purification

The UltraClean™15 DNA Purification Kit and Roche Agarose Gel DNA extraction kit were used to purify DNA from agarose gel and PCR products. These kits utilise silica beads that bind DNA in the presence of a chemotropic salt. DNA bound to the silica is washed free of impurities and then resuspended in water or buffer. The kits were used as per the manufacturer's instructions. Purified DNA was eluted in TE buffer.

2.9 Sequencing

DNA sequencing was performed at Canterbury Sequencing (School of Biological Science University of Canterbury) with a capillary ABI3100 Genetic Analyzer from Applied Biosystems Inc., using a procedure based on the Sanger chain-termination protocol. Purified DNA (Section2.8) was either sent directly for sequencing or the "BigDye Terminator" sequencing reaction was performed and the product sent for analysis.

2.9.1 "BigDye Terminator" sequencing reaction

The Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit was used to perform the sequencing reaction following the manufacture's instructions. BigDye Master Mix (Section2.9.1) was adjusted for template concentration and the extension temperature matched to specific primers.

Sequencing product was purified through Sephadex resin (Appendix 6.2.9). Sephadex (500 µl per sample) was added to a Whatman Ultrafilter plate and spun at 750 × *g*, 5 min. Flow through was discarded and the plate spun at 750 × *g*, 3 min. The sequencing sample was added to the column and spun through to a collection tube at 750 × *g*, 5 min.

2.10 Quantitative Real-time Polymerase Chain Reaction

Relative gene expression was measured using the quantitative real-time polymerase chain reaction (qPCR). This reaction utilises SYBR green which binds double strand DNA and fluoresces allowing the quantification of double stranded DNA. The accumulation of the PCR product is measured in terms of Relative Florescence Units (RFU).

2.10.1 Reaction protocol

Reactions volumes of 20 µl were used, consisting of 7 µl nanopure water, 10 µl 2×SYBR green

qPCR reaction buffer (Appendix 6.2.11), 1 µl cDNA and 1 µl of both the forward and reverse primers. The thermo-cycle consisted of an initial heating step of 10 min at 95°C which was followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and 80°C for 30 s. Fluorescence was measured at 80°C to eliminate contaminating fluorescence from dimers. Following the amplification cycles, a melting curve was generated by first heating the sample to 95°C for 1 minute, 55°C for 30 s then raising the temperature to 95°C, measuring the fluorescence every 0.5°C. All reactions were carried out using a Stratagene Mxpro-Mx5005P Thermocycler.

2.10.2 Gene expression data analysis

Data analysis was based on the methods of Pfaffl (2001). The expression of target genes is calculated relative to constitutively expressed reference genes. Threshold cycle (*Ct*) values were measured at 2000 fluorescent units. This fluorescence threshold was above the background noise levels and centrally located in the exponential amplification zone.

Efficiency (*E*), the rate of increase in DNA per cycle, was determined by the serial dilution method (Ramakers et al., 2003). Throughout the exponential expansion phase of the reaction, there is a linear relationship between the LOG of the florescence (*f*) and the Cycle number (*C*) described by the equation $LOG\ (f) = m\ (C) + b$ PCR efficiency was calculated from the slope of the line *m* by the following equation:

$$E = 10^{(m)} \tag{1}$$

Relative gene expression was determined by calculating the average relative expression of target genes to the expression of each of the reference house keeping genes, actin (*ACT*) and glyceraldehyde-3-phosphate dehydrogenase (*GAP*), Using the relative expression ratio method of Pfaffl (2001).

$$R = \frac{\left(E_{target}\right)^{\left(\overline{X}(C)_{target} - \overline{X}(S)_{target}\right)}}{\left(E_{refrence}\right)^{\left(\overline{X}(C)_{refrence} - \overline{X}(S)_{refrence}\right)}} \tag{2}$$

Where $\bar{x}(C)_{target}$ and $\bar{x}(C)_{refrence}$ are the average Ct values of the control for the target and reference

genes, $\bar{x}(S)_{target}$ and $\bar{x}(S)_{refrence}$ are the average Ct values of the subject for the target and reference genes and *E* is the respective PCR efficiency for the target and reference genes.

Chapter 3

Development of a micro-scale chlorophyll analysis

Spectrophotometry is commonly used for measuring chlorophyll levels. Traditionally, analysis has been carried out in 10 mm cuvettes with volumes of 0.2 - 2.0 ml. The NanoDrop Spectrophotometer requires a sample volume of 1-2 µl which provides the opportunity to analyse far smaller samples. Here an assay is described for the spectral determination of chlorophyll using the NanoDrop Spectrophotometer that allows the determination of chlorophyll from part samples of clover leaves while leaving sufficient tissue for multiple RNA extractions

3.1 Introduction

Chlorophyll levels are a standard measure in many plant studies. The most commonly used methods for determining chlorophyll concentrations are assays based on the Arnon assay (Arnon, 1949). The equations presented in the original Arnon assay were based on inaccurate spectrophotometric data. Subsequently a number of authors have offered corrections to these equations (Inskeep and Bloom, 1985; Porra et al., 1989; Wellburn, 1994; Porra, 2002).

The original Arnon assay was developed with 80% acetone. However, there are a number of problems with assaying chlorophyll in acetone. The main problems with acetone relate to its chlorophyll extraction efficiency (Lichtenthaler, 1987) and evaporation rate. Variations in water content and pH also affect the specific absorption coefficients of chlorophyll *a* and *b* (Wellburn, 1994).

Moran and Porath (1980) developed a chlorophyll assay using DMF. This was further optimised by Inskeep and Bloom (1985), Porra et al. (1989) and Wellburn (1994). DMF has a number of advantages over acetone. Chlorophyll is highly soluble in DMF enabling chlorophyll to be extracted from intact tissues eliminating grinding steps (Moran and Porath, 1980), chlorophyll is stable in DMF (Moran and Porath, 1980; Inskeep and Bloom, 1985), and DMF is considerably less volatile than acetone.

Traditionally, spectrophotometry has been carried out in 10 mm path length cuvettes which require

samples between 200 µl and 2 ml. The NanoDrop™ spectrophotometer takes measurements across path lengths of 1.0 mm and 0.2 mm, with samples sizes between 2 µl and 1.0 µl. By measuring the absorbance across two path lengths the NanoDrop can measure up to the 10 mm path length equivalent of 75 Absorbance units compared to 2 Absorbance units with traditional spectrometers.

3.2 Materials and methods

3.2.1 Comparison between the NanoDrop™ and the Novaspec II

A bulk chlorophyll extract was produced by immersing 1.0 g of fresh clover leaves in 10 µL of DMF in a 20 ml tube which was wrapped in tinfoil and then incubated at 4°C overnight. The following day the chlorophyll extract was decanted into micro-centrifuge tubes and spun at 2.3×10^5g for five min to pellet any suspended particles. The supernatant was decanted into a foil wrapped 20 ml tube.

The absorbance of the undiluted chlorophyll extract (2 µl samples) was measure at 664 and 647 nm with the NanoDrop spectrophotometer. A ten fold dilution of the extract was prepared to bring it within the reading range of the Novaspec II. The absorbance of the diluted extract was measured with the Novaspec II using 2 ml samples with a 10 mm path length quartz cuvette and with the NanoDrop using 2 µl samples. Chlorophyll concentrations were calculated using the equations of Wellburn (1994).

$$Chl_{total} = 7.12A_{664} + 18.12A_{647}$$

3.2.2 Chlorophyll determination during leaf development

Clover plants were grown using the method of Hunter et al. (1999). Leaves were excised from the petiole and frozen under liquid nitrogen in 1.5 ml micro-centrifuge tubes. Frozen leaf tissue was ground in micro-centrifuge tubes with a plastic pestle, taking care to avoid moisture condensation on the tissues. Between 9 and 33 µg of ground tissue were weighed into micro-centrifuge tubes, pre-chilled in liquid nitrogen, followed by the immediate addition of 200 µl of DMF. Samples were wrapped in tin foil and incubated in the refrigerator at 4°C overnight.

The following day tissues were centrifuged at 2.3×10^5g for 5min to pellet the ground leaf tissue. Three samples of 2 µL were taken from each tube and their absorbance measured at 664 and 647 nm using the NanoDrop spectrophotometer. Chlorophyll concentrations were calculated with the

equations of Wellburn (1994).

3.3 Results

3.3.1 Spectrometer comparison

The Novaspec II and the NanoDrop spectrophotometers produced slightly different estimates of chlorophyll concentration (Figure 3.1) with the Novaspec II reading 5% higher than the NanoDrop. When re-measuring the same sample the repetition error was lower for the Novaspec II (SE = 0.2), the NanoDrop decreased in accuracy when reading at lower concentrations sample (undiluted; SE = 1.0, diluted $\times 10^{-1}$; SE = 2.5). Dilutions of chlorophyll extract showed a linear relation ship to chlorophyll content for the NanoDrop across the full range tested, whereas the Novaspec II response was only linear between 10 µg/ml and 40 µg/ml of chlorophyll (Figure 3.2).

3.3.2 Developmental leaf series

Clover plants trained as a single stolon over a dry matrix exhibit the full spectrum of leaf development, with expanding leaves at the tip and senescent leaves at the basal end of the stolon (Figure 3.3). Total chlorophyll was used to gauge leaf maturity.

Chlorophyll content from the individual leaves of 6 plants was determined with the NanoDrop spectrometer (Figure 3.4). Chlorophyll content increased during leaf development until the leaves were fully expanded around leaf 3, chlorophyll content remained constant in mature leaves until the on set of senescence at leaf 11, thereafter chlorophyll content steadily declined.

3.4 Discussion

The large reaction volumes required by the traditional spectrophotometers have limited chlorophyll analyses of small or precious samples. Analysing a 1 µl 10% (w/v) sample in the NanoDrop will require only 0.1mg of tissue whereas a 1 ml sample analysed traditionally would require 10 mg of tissue. With the NanoDrop the limiting factor moves from the spectrometer to accurately weighing small samples.

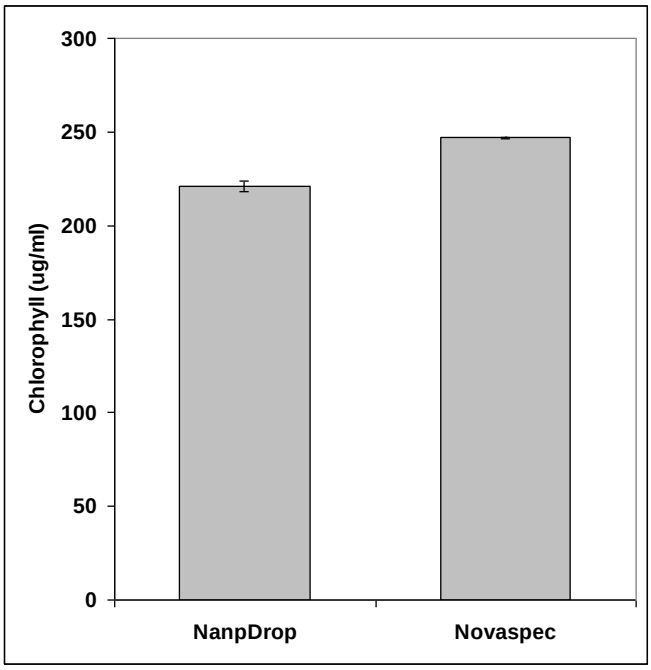


Figure 3.1 Chlorophyll measured with the NanoDrop and Novaspec

Chlorophyll concentrations determined using the Novaspec II and NanoDrop spectrophotometers. Optical densities of a 10-fold diluted chlorophyll extract was measured with both the NanoDrop and the Novaspec II. Results are mean values ± S.E, n=9.

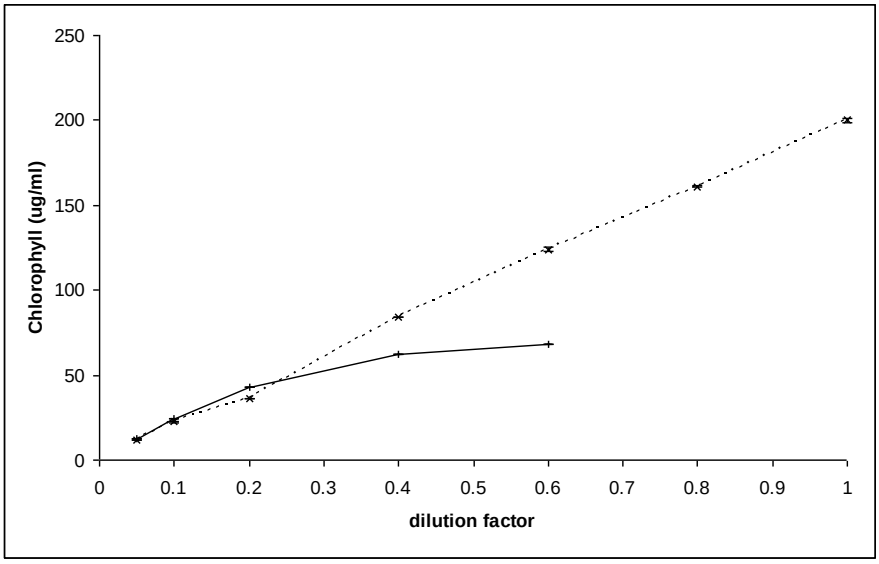


Figure 3.2 Dynamic range of chlorophyll determination

Chlorophyll concentrations for a series of dilutions of raw chlorophyll extract determined using the Novaspec II (solid line) and NanoDrop (dashed line) spectrophotometers. Results are mean values ± S.E, n=3. Error bars are smaller than the symbols.

Figure 3.3 A clover stolon

A single clover stolon showing the full range of leaf development.

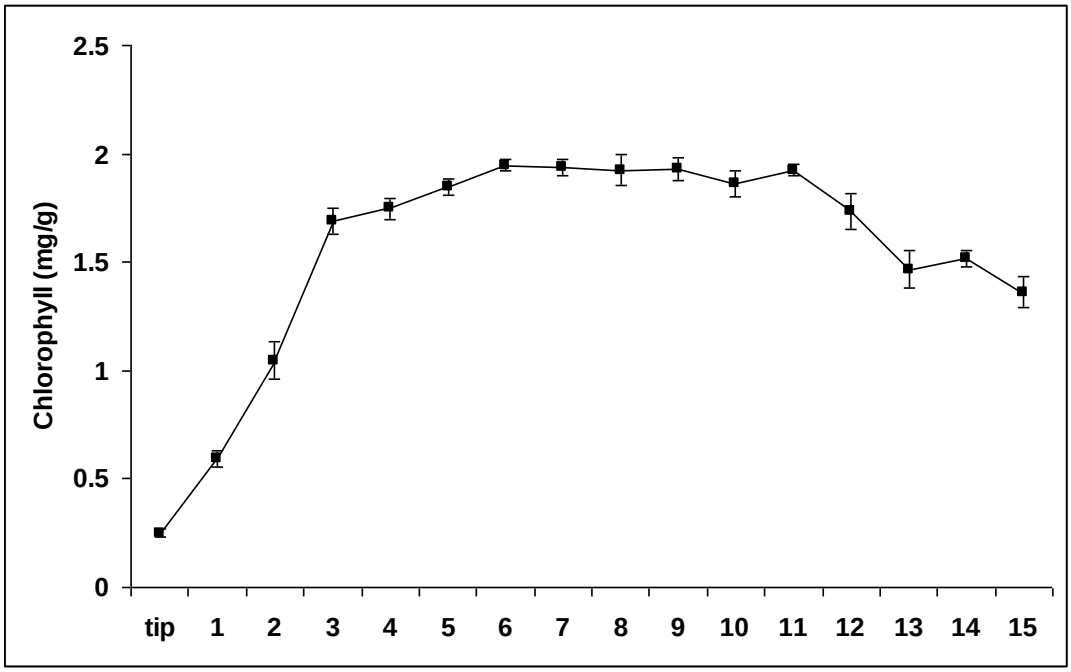


Figure 3.4 Chlorophyll throughout leaf development

Total chlorophyll in white clover leaves throughout leaf development, measured using the NanoDrop spectrophotometer (Mean values ± S.E, six leaves with three technical replicates).

There was a 5% difference in estimates of chlorophyll between the Novaspec II and the NanoDrop. It was not immediately apparent as to whether the chlorophyll concentration was overestimated when the Novaspec II was used or whether the chlorophyll concentration was underestimated when the NanoDrop was used. If the NanoDrop is to be used to determine absolute chlorophyll levels a set of chlorophyll equations specific to the NanoDrop should be developed using the methods of Lichtenthaler et al. (1987) and Porra (1989).

When analysing the same sample the Novaspec II provided more consistent results than the NanoDrop. Chlorophyll estimates made with the NanoDrop varied by 3-6% (depending on concentration) compared to 0.5% with the Novaspec II. However the large absorbance range of the NanoDrop allows a far wider range of sample concentrations to be accurately measured than the Novaspec II. Consequently the NanoDrop reduces the requirement to dilute samples for analysis, eliminating the associated error, saving time and improving the relative accuracy of the NanoDrop.

Using the NanoDrop, the chlorophyll content from part samples of individual clover leaves could be determined, leaving sufficient leaf tissue for two or more RNA extractions. Chlorophyll levels increased during leaf development and began to decline at the onset of senescence around leaf twelve. The chlorophyll levels and pattern of development are consistent with previous studies (Hunter et al., 1999; Yoo et al., 2003).

In summary, the NanoDrop Spectrophotometer provides a fast and efficient means of measuring relative chlorophyll from very small sample sizes. The NanoDrop is not as accurate as the Novaspec II but its use eliminates the need to dilute samples. Caution should be used when comparing absolute chlorophyll levels measured with the NanoDrop with those from other spectrometers without developing NanoDrop specific chlorophyll equations.

Chapter 4

Gene isolation and expression

4.1 Gene identification

4.1.1 *IPT* gene identification

A thorough search of the public sequence databases failed to identify any clover *IPT* sequences. Previous studies have identified four *IPT* genes in *Lotus japonicus*, two *IPT* genes in *Pisum sativum* and one *IPT* gene in *Glycine max* (Appendix 6.1.6). A blast search of legume EST databases identified a number of EST's with homology to IPT (Appendix 6.1.3).

The phylogenetic relationships between sequences were estimated and degenerate PCR primers were designed for clades within the phylogeny. PCR with degenerate *IPT* primers (Table 4.1) and *Trifolium repens* cDNA amplified a sequence of approximately the expected size (Figure 4.1). Direct sequencing of the PCR product with the degenerate IPT primers identified one putative *Trifolium repens* IPT gene (Appendix 6.4.1). It was named *TrIPT1*. Gene specific primers were developed for *TrIPT1* (Table 4.1). RT-PCR with two pairs of the specific *TrIPT1* primers amplified products of the expected size (Figure 4.1) plus bands of other sizes.

Six months into the project access was obtained to the Pastoral Genomics sequence database. The Pastoral Genomics database was searched with BLAST and a further four putative *IPT* genes were identified: *TrIPT2, TrIPT3, TrIPT4* and *TrIPT5* (Appendix 6.1.1). PCR primers were developed for each gene (Table 4.1). RT-PCR with gene specific primers amplified products of the expected size for *TrIPT3* (Figure 4.2) and *TrIPT5* (Figure 4.3). Sequencing of the *TrIPT3* and *TrIPT5* PCR products confirmed the specificity of the primers for their targets *TrIPT3* (Appendix 6.4.2) and *TrIPT5* (Appendix 6.4.3). RT-PCR and sequencing failed to detect the expression of either *TrIPT2* (Appendix 6.4.4) or *TrIPT4* (Appendix 6.4.5).

PCR with the primers TrCKX2f2 and TrCKX2r2 produced a band at an expected size of 1000 bp (Figure 4.5). Sequencing of the 1000 bp band identified a fragment of the white clover mosaic virus genome (Accession: X16636).

Table 4.1 PCR primers

Gene	Primer name	
TrIPT1	TDipt4 F1	GCWACMGGRACRGGRAAGTC
	TDipt4 R1	RCCWCCRRCGATGATYGGAAG
	TrIPT1F1	ACAAACAAAATCACCAAAGAAGAAC
	TrIPT1F2	CTTAGCAAATTATTTCCCATCAGA
	TrIPT1R1	AGATTGGAAGATGTTCGCGG
	TrIPT1R2	GCGAAAATCGTTGGAAGTGA
TrIPT3	TrIPT3F1	TATATTTTCGGGTGCTGTTTCT
	TrIPT3R1	CGGTGAAAAATGTGTTGCTATATC
TrIPT5	TrIPT5F2	ATGTGTCTTTGCCTATTCTATTTC
	TrIPT5R2	TCAATTTTGTTCATCTTCCATCC
TrCKX1	TDckx2f1	CATGGHCAYTCMCTYCAAGG
	TDckx2R1	ATCACAAASCCTTCNABGTAATC
	TrCKX1F1	TCACGGAAACGACACAGAATC
	TrCKX1R1	AACCCGAGATAATCAGTCCAC
TrCKX2	TrCKX2F1	CACCCAATAGAAGCACTAACACA
	TrCKX2F2	AAACCTTATTCGTGACCCATTG
	TrCKX2R1	TACATACCCGTAACAACATCCA
	TrCKX2R2	GGTTTGTCCACTAATACCAGCA
TrCKX6	TrCKX6F1	TGGGTTAGCACCTATGTCTTGG
	TrCKX6R2	CAAGACCACCAAGAACAGCATG
TrCKX7	TrCKX7F1	TCATTGGTTACCCCAGAGGAAG
	TrCKX7F2	GCATTCCTATCCTCTGCAGTCC
	TrCKX7R2	AGCAGTGCCAGTGGGTCATAG
	TrCKX7R1	TGCCTTTTGAAAGATCCTATGG
TrACT	TrACTF2	ATGGARAAGATYTGGCATCACA
	ACTR2	RTGRSWSACACCATCACCAGA
TrGAP	TrGAPF2	GGAATCGTTGAGGGTCTTATGA
	TrGAPR2	CCTCAGAYTCCTCCTTGATAGCA
TrPP2	TrPP2F2	TVCCTGAAGATGTTCGGCTG
	TrPP2r2	GGTGCCATTCCCATTATWACTG

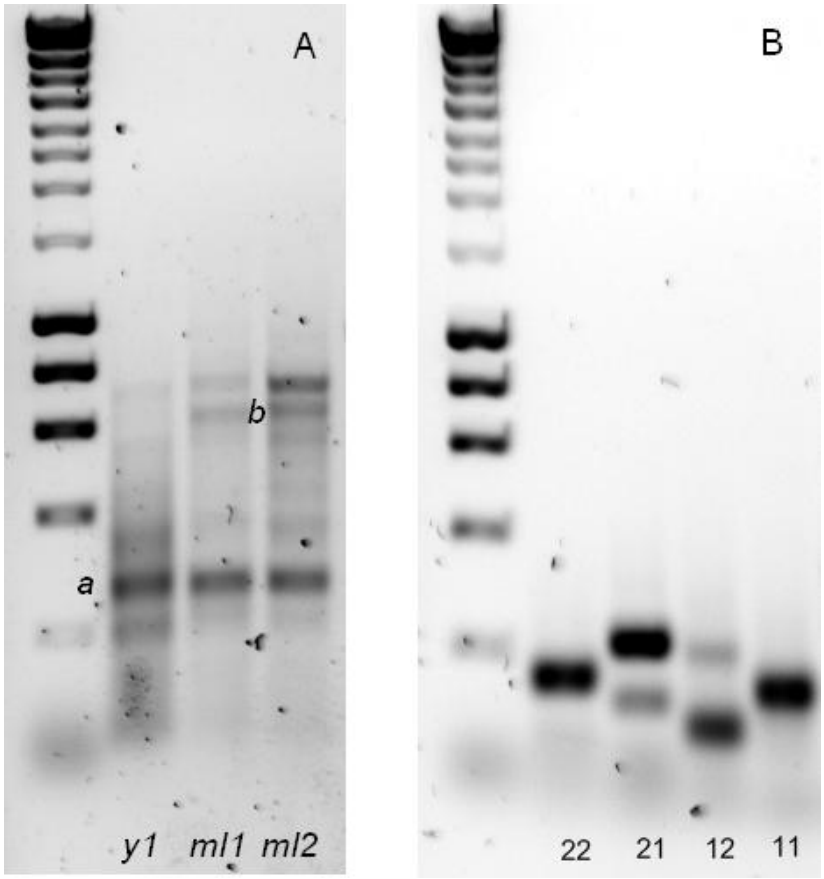


Figure 4.1 PCR results *TrIPT1*

A: PCR results from TDIPT4 F1 and R1 with cDNA from young leaves (yl) and two mature leaves (ml1 and ml2). Band (a) is at the expected product size of 270bp. Sequencing and BLAST analysis revealed a putative *IPT* gene. Sequencing of band (*b*) revealed a putative GDP-mannose 3',5'-epimerase gene.

B: The PCR products for *IPT1* produced with specific *TrIPT1* primers and mixed *T. repens* cDNAs. TrIPT1f2/TrIPT1r2 (22) and TrIPT1f1/TrIPT1r1 (11) produced single bands at their expected sizes of 168 bp and 150 bp respectively. TrIPT1f2/TrIPT1r1 (21) and TrIPT1f1/TrIPT1r2 (12) both produced two bands, one the expected size (223 bp and 95 bp respectively) and a second unexplained band.

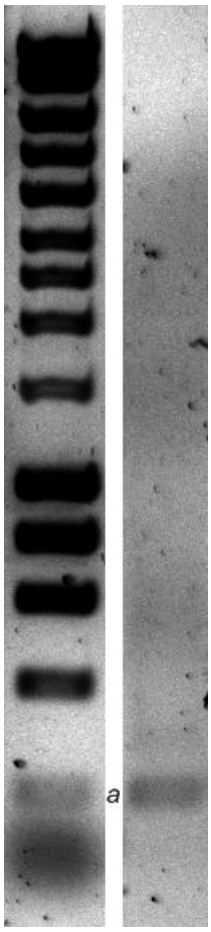


Figure 4.2 PCR results *TrIPT3*

PCR products for *TrIPT3*, PCR reaction performed with TrIPT3F1/TrIPT3R1 primers and senescent leaf cDNA produced a band (*a*) at the expected size (190 bp). The product identity was confirmed by sequencing.

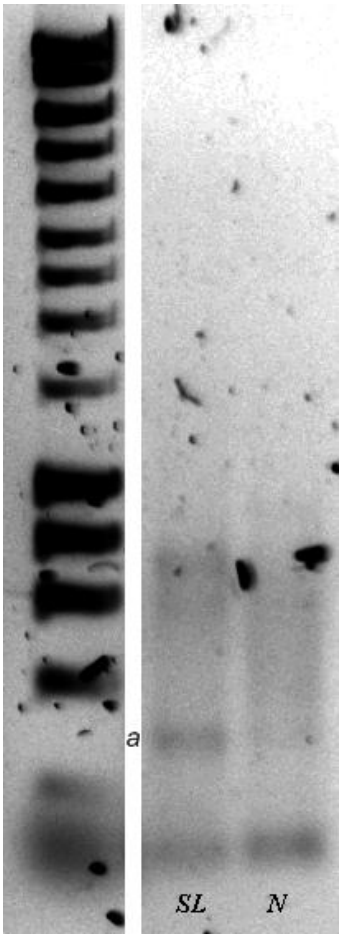


Figure 4.3 PCR results *TrIPT5*

RT-PCR products for TrIPT5 (320 bp), produced using TrIPT3rF2/TrIPT5R2 primers with senescent leaf cDNA (sl) and first node cDNA (n). Sequencing confirmed the product as *TrIPT5*.

4.1.2 *CKX* gene identification

An extensive search of the public sequence databases identified a 450 bp *Trifolium repens* micro satellite sequence with homology to the 5' end of the *Pisum sativum* gene *PsCKX1* (Expect = 1e-163) and *Arabidopsis thaliana AtCKX7* (Expect = 6e-36). Two similar legume sequences were identified from *Glycine max* and *Medicago truncatula* (Appendix 6.1.4).

Degenerate primers were designed for these sequences (Table 4.1). PCR with the degenerate *CKX* primers and cDNA derived from *Trifolium repens* L. genotype 10F amplified a sequence of approximately the correct length (Figure 4.4). Direct sequencing of the PCR product identified a 714bp sequence from the 5' end of a CKX gene (Appendix 6.4.7). This gene was named *TrCKX1*.

Data mining of the Pastoral Genomics database with BLAST identified a further six sequence fragments for putative CKX genes that were named *TrCKX2*, *TrCKX3*, *TrCKX4*, *TrCKX5*, *TrCKX6* and *TrCKX7* (Appendix 6.1.2). BLAST analysis identified a 96 bp intron in the *TrCKX6* gene fragment (Appendix 6.4.9). Specific PCR primers were designed for all 7 putative *Trifolium repens* CKX genes (Table 4.1). RT-PCR with *Trifolium repens* cDNA amplified products for *TrCKX1* (Figure 4.4), *TrCKX2* (

Figure 4.5), *TrCKX6* (Figure 4.6) and *TrCKX7* (Figure 4.7). The PCR products were sequenced to confirm the identity of the target gene. The sequence results for *TrCKX1* (Appendix 6.4.7), *TrCKX2* (Appendix 6.4.8) and *TrCKX7* (Appendix 6.4.10) matched their target genes although a number of ambiguous sites were present implying the presence of duplicate copies of the gene as expected in an allotetraploid.

Sequencing of TrCKX6 (Appendix 6.4.9) PCR products produced a sequence with a lot of ambiguity. Close inspection revealed that both cDNA transcripts and genomic DNA had been sequenced. The larger of the two band was due to the presence of an intron in the sequence. Aside from the genomic contamination, with the high degree of ambiguity observed, it appears that these primers are not specific to *TrCKX6* possibly amplifying multiple CKX gene family members. RT-PCR and sequencing failed to detect the expression of *TrCKX3* (Appendix 6.4.11) *TrCKX4* (Appendix 6.4.12) or *TrCKX5* (Appendix 6.4.13).

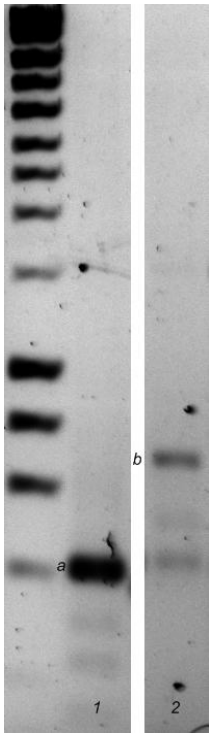


Figure 4.4; PCR results *TrCKX1*

Rt-PCR products for *TrCKX1* produced with degenerate *CKX* primers and specific *TrCKX1* primers. TrCKX1f1/TrCKX1r1 (lane 1) produced a single band (*a*) at the expected 423 bp. TdCKX2f1/TdCKX2r1 (lane 2) produced a product (*b*) at 720 bp. Both reactions were performed with mixed tissue *T. repens* cDNA. Sequencing showed both *a* and *b* to be fragments of the same CKX gene.

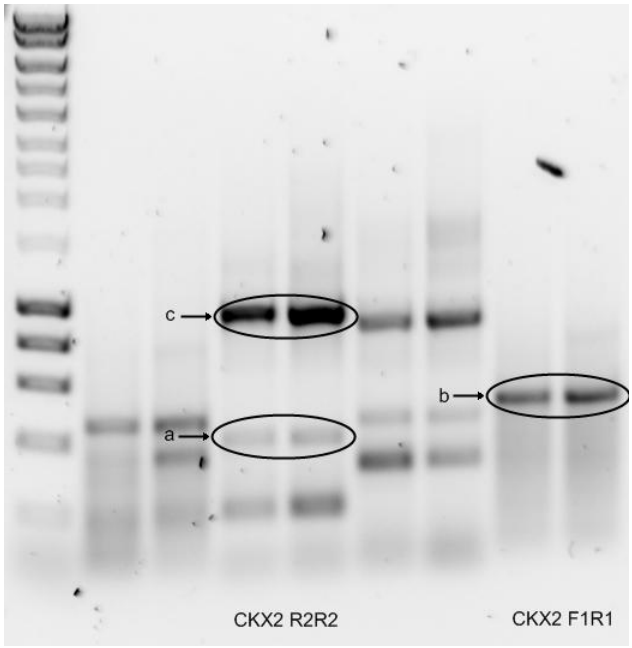


Figure 4.5; PCR results *TrCKX2*

PCR products for *TrCKX2* using all CKX2 primer combinations and green senescent leaf (left) and yellow senescent leaf (right) cDNAs. The TrCKX2f1/TrCKX2r1 primers (CKX2F1R1) produced a single band (b) at the expected size of 551 bp. The TrCKX2f2/TrCKX2r2 primers produced two bands, one at the expected 429 bp (a) and second sequence at 1000 bp (c). The three bands were sequenced; *a* and *b* matched target *TrCKX2* sequence and *c* matched white clover mosaic virus (Accession: X16636).

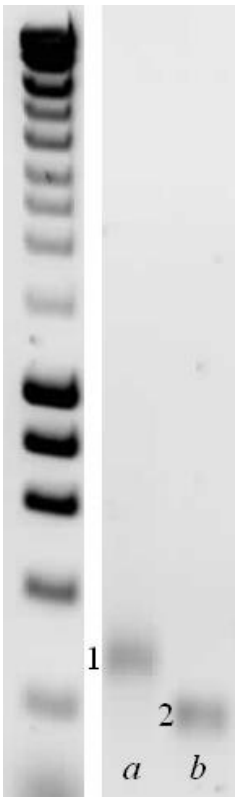


Figure 4.6; PCR results *TrCKX6*

The PCR products for *TrCKX6*. the TrCKX6 primers TrCKX6f1/TrCKX6r2 showing the genomic product (lane *a*) and and cDNA product (lane *b*) with products at the expected 300 bp (1) and 206 bp (2). Sequencing confirmed both products.

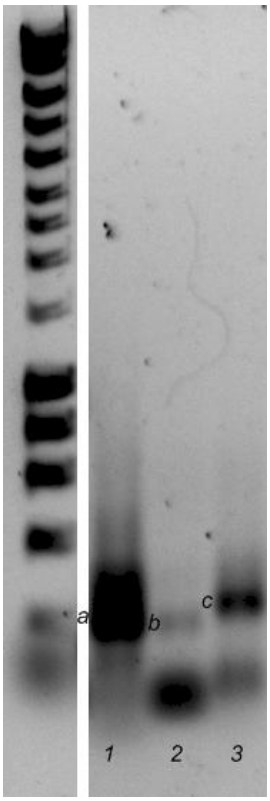


Figure 4.7; PCR results *TrCKX7*

The PCR products for *TrCKX7* using the primers TrCKX7F1/TrCKX7R2 (*1*), TrCKX7F2/TrCKX7R1 (*2*) and TrCKX7F1/TrCKX7R1 (*3*) with senescent leaf cDNA. All three produced products at the expected sizes of 259 bp (*a*), 251 bp (*b*) and 290bp (*c*) respectively. Sequences confirmed all three products as *TrCKX7*

4.1.3 Glyceraldehyde 3-phosphate dehydrogenase (*GAP*)

A BLAST search of the Pastoral Genomics database found a genomic DNA sequence containing part of the *GAP* gene sequence (Appendix 6.1.5). This genomic sequence is punctuated by three introns (Appendix 6.4.16.1). PCR primers were designed to amplify a 298 bp sequence fragment. RT-PCR produced a product at the expected size (Figure 4.8). Sequencing confirmed the specificity of the primers and the placement of the three introns in the genomic sequence (Appendix 6.4.16.2).

4.1.4 Actin (*ACT*)

A search of the public databases identified a partial sequence for a *Trifolium repens Actin* cDNA transcript (Appendix 6.1.5) and a full length *Trifolium pratense Actin* transcript (Appendix 6.1.5). A BLAST search of the Pastoral Genomics database identified the genomic *Actin* sequence (Appendix 6.1.5) containing three exons (Appendix 6.4.15.1). PCR primers were designed for these sequences (Table 4.1). RT-PCR (Figure 4.8) and sequencing confirmed the specificity of the primers (Appendix 6.4.15.2). The sequence traces contain a number of ambiguous sites. This is likely the result of the primers amplifying two or more members of the *Actin* gene family.

4.1.5 Protein phosphatase 2 (*PP2*)

Medicago sativa and *Pisum sativum PP2* genes (Appendix) were pulled from public sequence databases. A BLAST search of the Pastoral Genomics database revealed a further 11 partial sequences for putative *Trifolium repens* PP2 genes (Appendix 6.1.5) *Trifolium repens* sequences were aligned with the *Medicago sativa* and *Pisum sativum* PP2 genes. The *Trifolium repens* sequences aligned well with the *Medicago sativa* and *Pisum sativum* genes at the amino acid level. There appear to be several closely related PP2 genes in *Trifolium repens*. PCR with primers designed for a conserved region of the aligned sequences (Table 4.1) produced a product at the expected 204 bp (Figure 4.8). The PCR product was sequenced and matched the target sequence (Appendix 6.4.17).

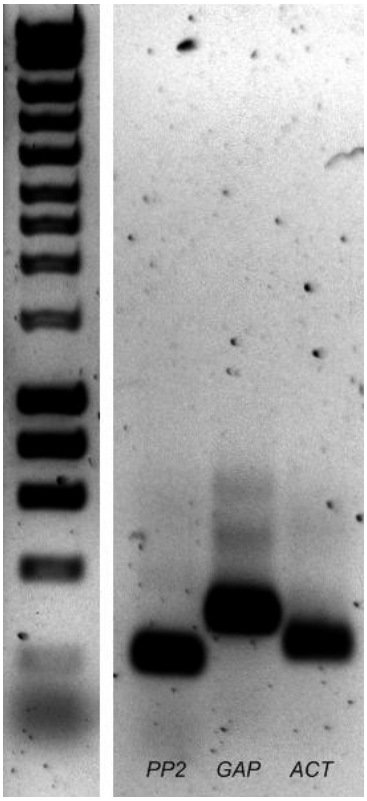


Figure 4.8 PCR results House keeping genes

The PCR products for *TrPP2*, *TrGAP* and *TrACT*. All three reactions were performed with cDNA derived from the first node. PCR with TrPP2F1/TrPP2R1 primers produced a product at the expected size (204 bp). The *GAP* primers TrGAPF2/TrGAPR2 produced a product at the expected size (296 bp) and the *ACT* primers TrACTF2/ACTR2 produced a product of the expected size (240 bp). All three were confirmed by sequencing the PCR products.

4.2 Phylogenetics

4.2.1 IPT Phylogeny

The phylogenetic relationship between the putative *T. repens IPT* genes and *A. thaliana, O. sativa, Z. mays* and legume *IPT* genes (Appendix 6.1.6) was determined using "Maximum Parsimony". Translated sequence data was aligned (Appendix 6.4.6) and a phylogenetic tree estimated using the Maximum Parsimony function in MEGA4 (Figure 4.9). Monocot and dicot *IPT* genes formed two distinct clades. *TrIPT1* grouped together with *LjIPT3* (Bootstrap support 87). *TrIPT2* is grouped together with *LjIPT1* (Bootstrap support 99). Both *TrIPT2* and *TrIPT5* are present in a larger clade with *LjIPT1, AtIPT6, AtIPT1* and *AtIPT4*. *TrIPT3* grouped closest to *PsIPT1*. *TrIPT4* is grouped together with *PsIPT2*, *GmIPT* and *LjIPT4* in a larger clade that includes *TrIPT3, LjIPT2*, *PsIPT1* and *AtIPT5*. *TrIPT5* was in a clade of its own branching from the base of the dicot IPT clade. There is only limited common sequence data between some of the *T. repens IPT* genes, which has affected the resolution of the phylogenetic tree. This is most evident with *TrIPT5*. The placement of *TrIPT5* within the tree is more a function of a lack of data than its evolutionary relationship.

4.2.2 CKX Phylogeny

The phylogenetic relationship between the putative *T. repens CKX* genes and *A. thaliana, O. sativa, Z. mays* and legume *CKX* genes (Appendix 6.1.7) was determined using "Maximum Parsimony". Translated gene sequences were aligned (Appendix 6.4.14) and a phylogenetic tree estimated using the Maximum Parsimony function in MEGA4 (Figure 4.10). *TrCKX1* is grouped with *PsCKX1* (Bootstrap support 99) in a well supported clade with *AtCKX7* and *OsCKX11*. *TrCKX2* is grouped together with *PsCKX* (Bootstrap support 75). *TrCKX3, TrCKX4* and *TrCKX5* formed a clade together with *AtCKC2*, *AtCKC3* and *AtCKC4*. *TrCKX6* branched from near the base of the tree. *TrCKX7* paired with *AtCKX1* (Bootstrap support 71) in a clade containing *AtCKX6, OsCKX4* and *OsCKX9*.

As with the *T. repens IPT* genes, only short fragments of the *T. repens CKX* genes have been sequenced and these gene fragments code for different regions of the gene with little of no overlap. This has affected resolution of the phylogenetic tree.

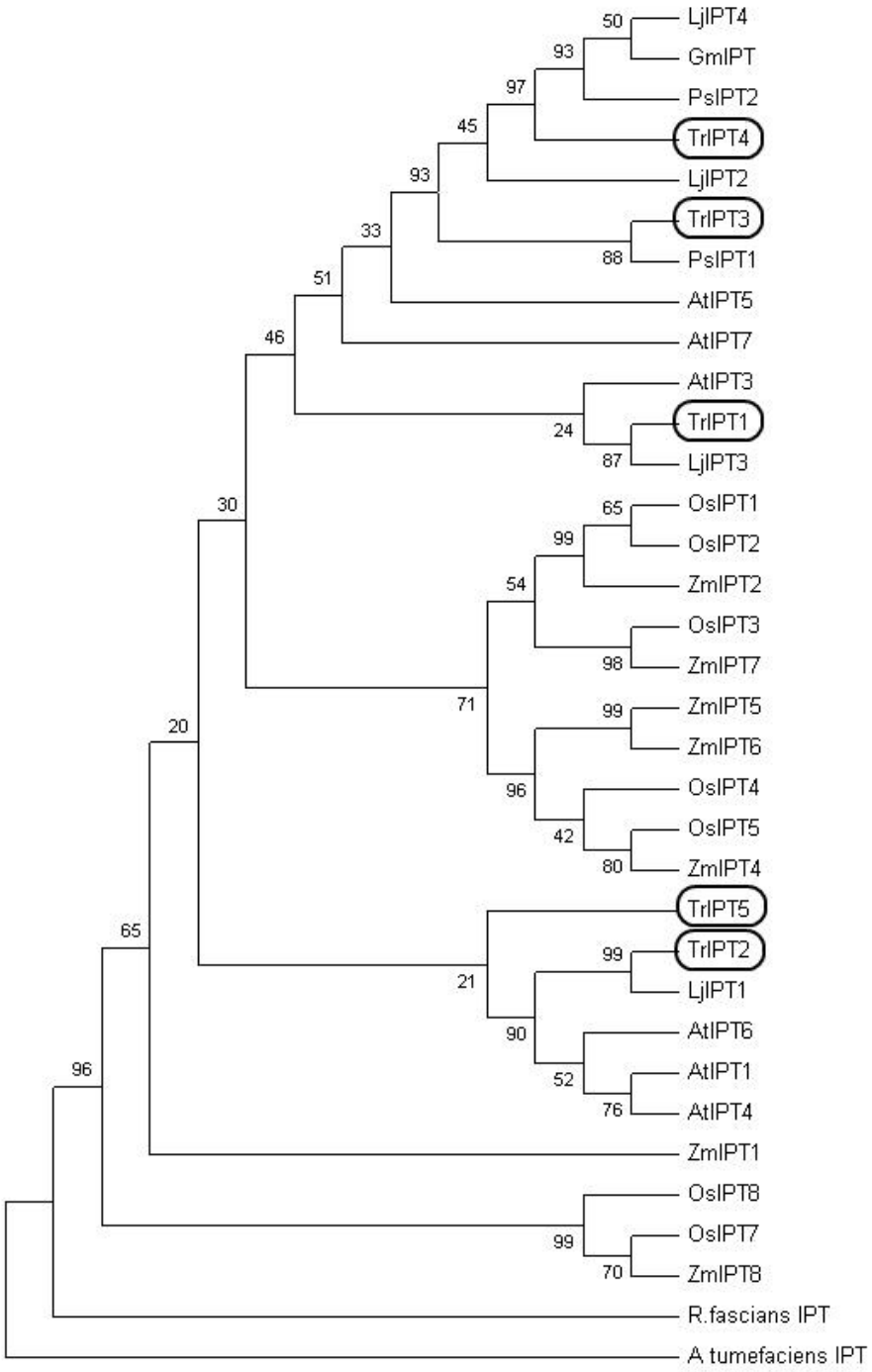


Figure 4.9 *IPT* Phylogenetic tree

IPT Phylogenetic tree; Tree showing the relationship between *Trifolium repens IPT* genes and the *Arabidopsis thaliana, Oryza sativa, Zea mays s* and legume *IPT* genes. Maximum parsimony tree rooted with *Rhodococcus fascians and Agrobacterium tumefaciens IPT genes.* Bootstrap tree branch support values shown (10000 bootstrap replications).

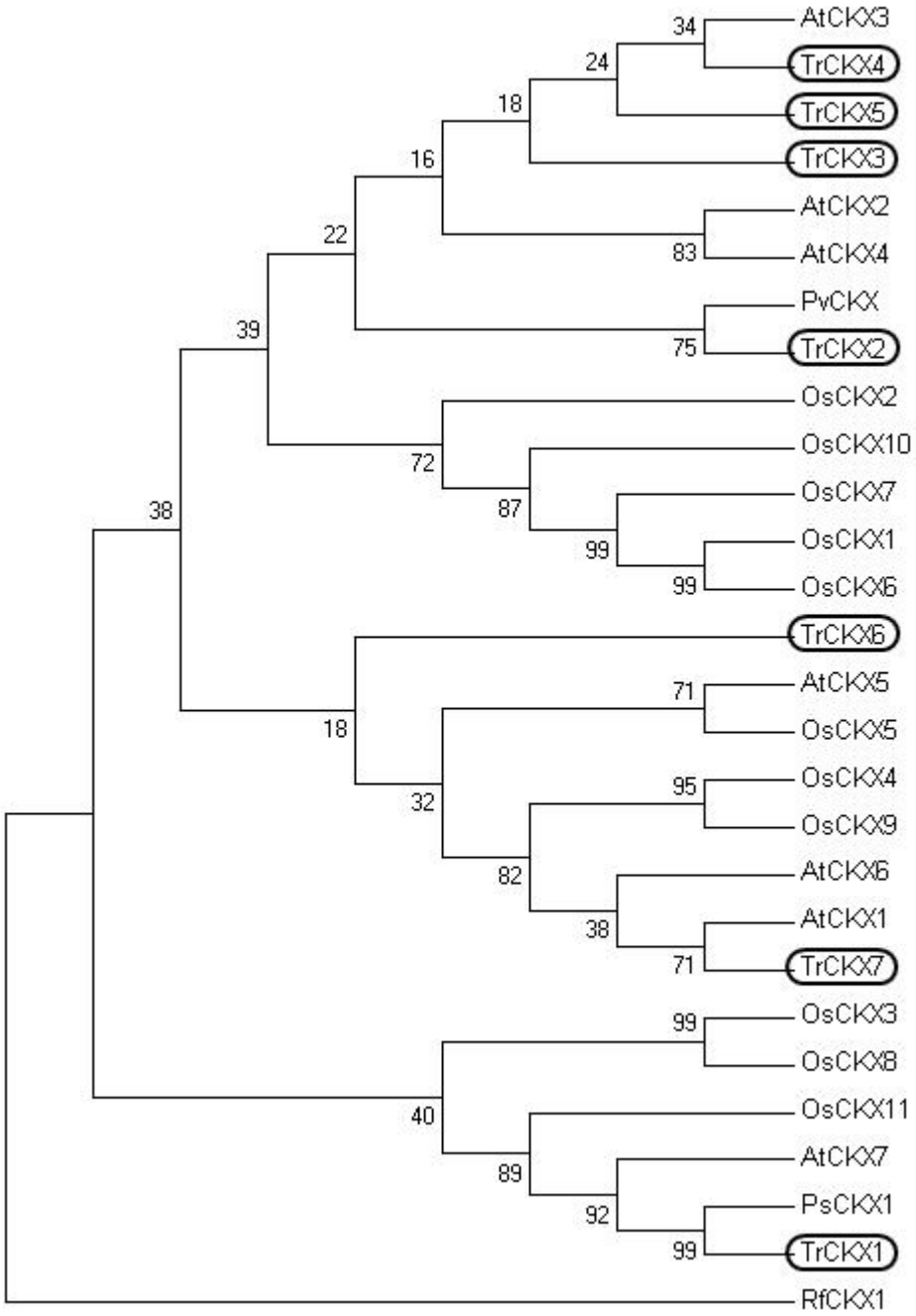


Figure 4.10 *CKX* Phylogenetic tree

Phylogenetic tree for *CKX*; Tree showing the relationship between *Trifolium repens CKX* genes and the *Arabidopsis thaliana , Zea mays* and legume *CKX* genes. Maximum parsimony tree rooted with *Rhodococcus fascians CKX gene*. Bootstrap tree branch support values shown (10000 bootstrap replications).

4.3 Gene expression

Using cDNA for PCR followed by sequencing *TrIPT1, TrIPT3, TrIPT5, TrCKX1, TrCKX2, TrCKX6* and *TrCKX7* we have shown to be expressed in the following tissues; TrIPT1, senescent leaves and mixed tissue cDNA; *TrIPT3*, senescent leaves; *TrIPT5*, node and senescent leaves; *TrCKX1*, node and senescent leaves; *TrCKX2*, senescent leaves; *TrCKX6*, node and leaves; *TrCKX7*, leaves.

Quantitative real time PCR (qPCR) with SYBR green detects any double stranded DNA in the reaction vessel, therefore primer specificity is critical. Primers were selected for qPCR from the primers successfully used for sequencing gene fragments. The following primers were selected for the target genes *TrIPT1*; TrIPT1F1/TrIPT1R1, *TrIPT3*; TrIPT3F1/TrIPT3R1, *TrIPT5*; TrIPT5F2/TrIPT5R2, *TrCKX1*; TrCKX1F1/TrCKX1R1, *TrCKX2*;TrCKX2F1/TrCKX2R1, TrCKX6; TrCKX6F1/TrCKX6R2, *TrCKX7*; TrCKX7F2/TrCKX7R1 and for the house keeping genes *GAP*; TrGAPF2/TrGAPR2, *PP2*; TrPP2F2/TrPP2R2 and *ACT*; TrACTF2/ACTR2 (Table 4.1). The primers were then tested for their suitability for qPCR with a range of cDNAs.

Two plants, Plant 5 (P5) and Plant 6 (P6), were selected from the six cultivated plants based on the developmental state of their first leaf and timing of senescence development. RNA was extracted from Plant 5 (P5) using the BP-10 Spin Column (Section2.4.2) and from Plant 6 (P6) with TRIzol (Section2.4.1).

4.3.1 Testing qPCR sensitivity

Melting curves give an indication of the composition of the PCR product. A single sharp peak indicates the presence of a single PCR product. The housekeeping genes *ACT*, *GAP* and *PP2* typically produced Ct values of between 16 and 22 (Figure 4.11, Figure 4.12 and Figure 4.13). The melting curves for both *ACT* (Figure 4.11) and *GAP* (Figure 4.12) PCR products produced sharp peaks at 85°C and 86°C respectively whereas the dissociation curve for *PP2* (Figure 4.13) contained multiple and variable tissue specific peaks. The irregular melting curves for *PP2* are indicative of both non-specific products and/or primer dimers. Therefore *PP2* was eliminated from further analysis.

Melting curves for the target gene PCR product produced mixed results with some reactions producing multiple or irregular peaks. Notably *TrIPT3* (Figure 4.14) produced double peaks with

some leaf cDNAs. The temperature of the melting curve peaks for *TrIPT5* varied within a 4°C band (Figure 4.15). *TrCKX2f1r1* produced two peaks (Figure 4.16). The melting curve peaks for *TrCKX6* were relatively broad (Figure 4.17). Some cDNAs produced irregular melting curve peaks with *TrCKX7* (Figure 4.18). *TrIPT1* (Figure 4.19) and *TrCKX1* (Figure 4.20) produced good singular sharp peaks.

Following on from the initial primer test qPCR was performed with clover leaf, root, nodule and stolon cDNAs to measure expression throughout leaf development and senescence. Ct values for the house keeping genes ACT and GAP were lowest in young leaves (Figure 4.21 and Figure 4.22). This is in line with the higher RNA yields achieved from these tissues, which have translated into higher transcript concentrations in the resulting cDNAs. The expression of *ACT* and *GAP* were closely aligned with their Ct values moving together with fluctuations in cDNA concentration. The movement of *ACT* and *GAP* together in each tissue, independent of the other genes, is in line with the expected pattern for constitutively expressed housekeeping genes. The Ct values recorded for the target genes remained relatively constant across all leaves and did not increase during early leaf development as observed for the housekeeping (Figure 4.21). *TrCKX1* and *TrCKX6* show the least variation between samples

Serial dilutions of template DNA were performed to determine the primer specific PCR efficiencies and to test the range of template concentration each qPCR assay can detect. Initially, qPCR was performed with serial dilutions of leaf cDNA. This did not produce the increase in CT values as expected. From the earlier gene isolation experiments it was evident that expression of many of the target genes was very low, often requiring two cycles of PCR to produce sufficient product for sequencing. When template concentrations are low any non specific product or primer dimers produced may obscure detection of the target gene product. To remedy this, qPCR was performed with serial dilutions of the gene specific PCR products that had been purified for sequencing. The purified product for *TrIPT1* and *TrCKX1* had been consumed and the sequencing results for *TrCKX6* showed this product was contaminated with other non specific products. Therefore analysis of their genes was not possible.

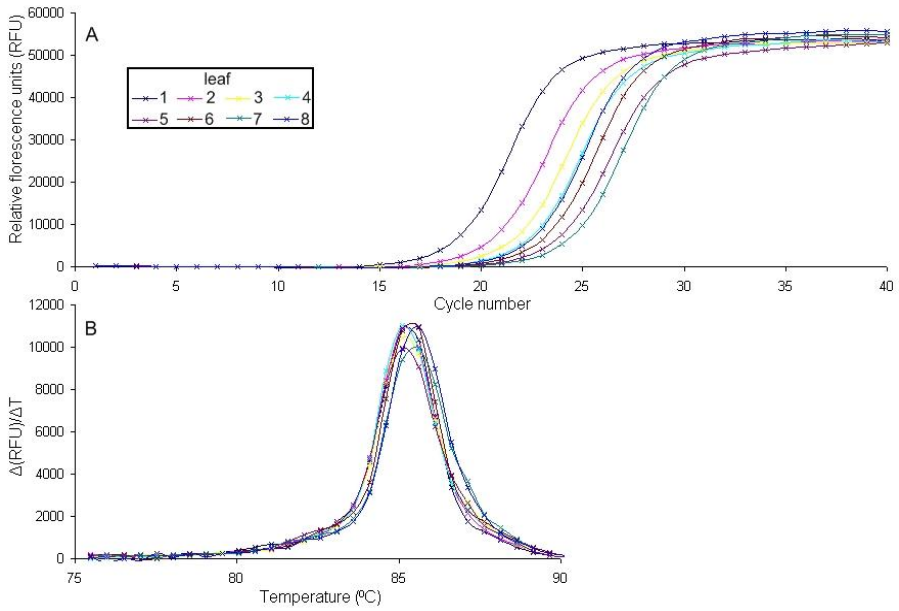


Figure 4.11 *ACT* amplification plot

ACT amplification plot (A) and melting curve (B). qPCR performed with leaf cDNAs and the *ACT* primers TrACTF2/ACTR2.

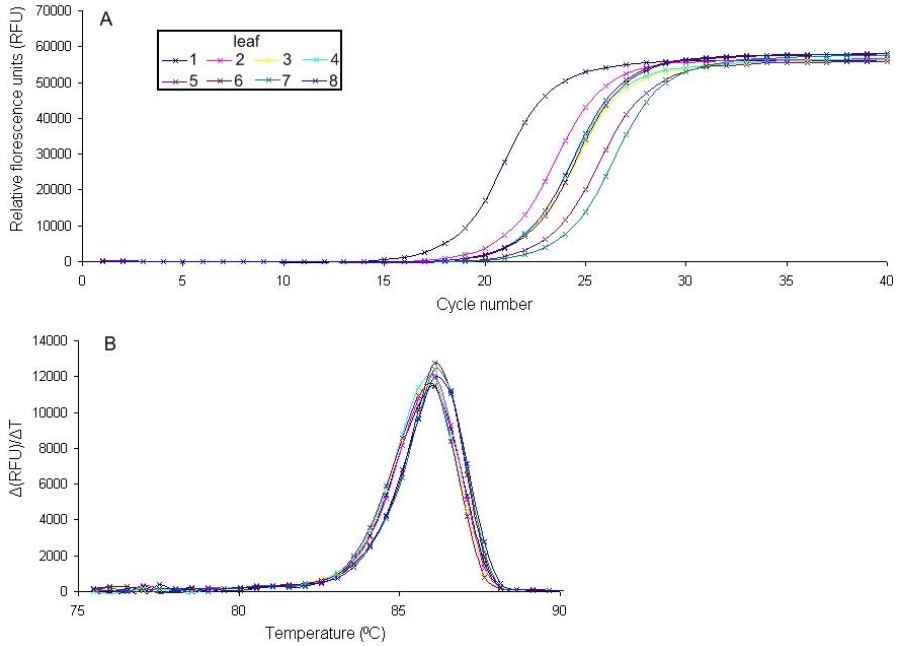


Figure 4.12 *GAP* amplification plot

GAP amplification plot (A) and melting curve (B). qPCR performed with leaf cDNAs and the *GAP* primers TrGAPF2/TrGAPR2.

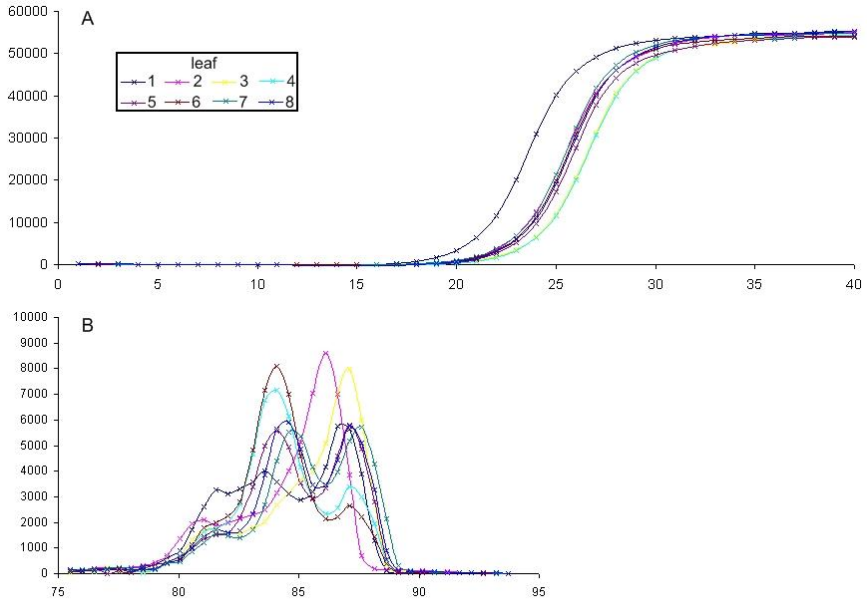


Figure 4.13 *PP2* amplification plot

PP2 amplification plot (A) and melting curve (B), qPCR performed with leaf cDNAs and the *PP2* primers TrPP2F2/TrPP2R2.

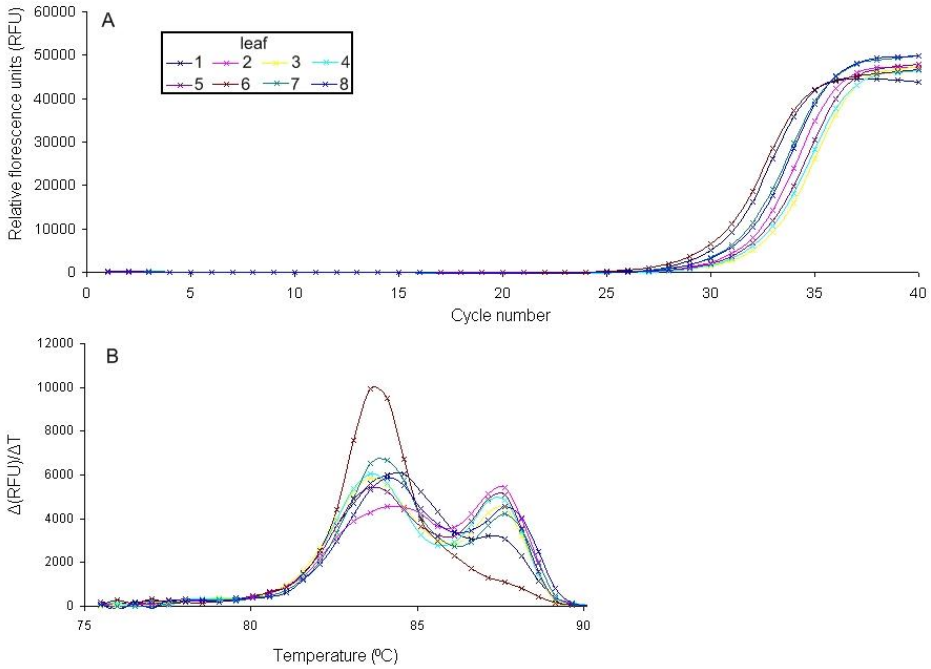


Figure 4.14 *TrIPT3* amplification plot

TrIPT3 amplification plot (A) and melting curve (B), qPCR performed with leaf cDNAs and the *TrIPT3* primers TrIPT3F1/TrIPT3R1.

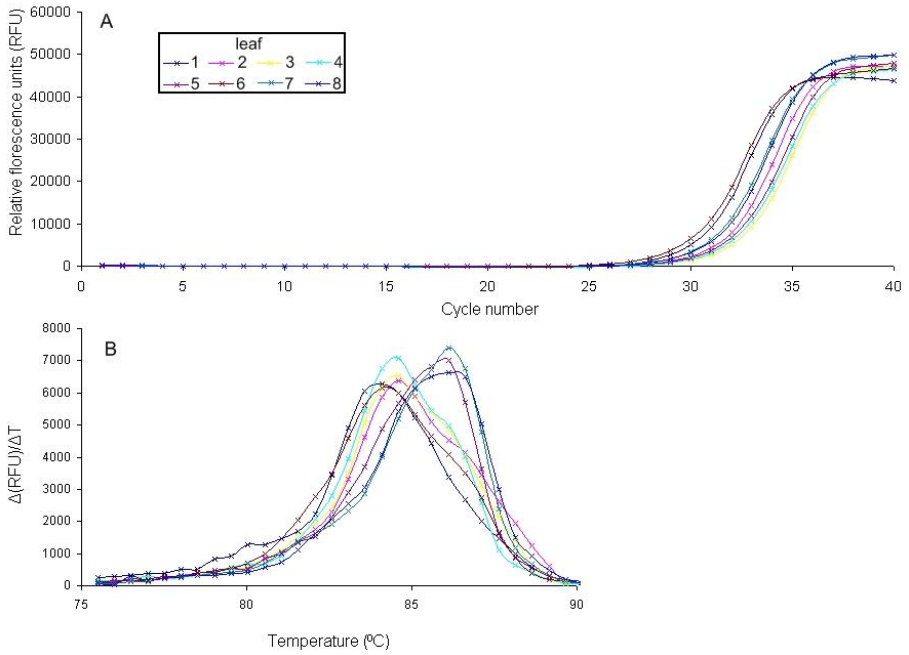


Figure 4.15 *TrIPT5* amplification plot

TrIPT5 amplification plot (A) and melting curve (B), qPCR performed with leaf cDNAs and the *TrIPT5* primers TrIPT5F2/TrIPT5R2.

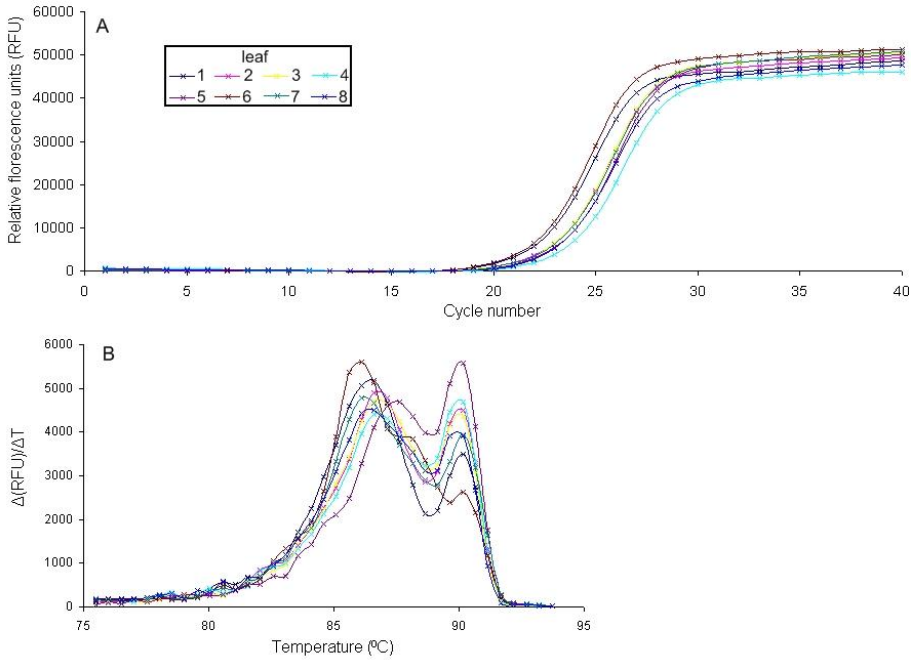


Figure 4.16 *TrCKX2* amplification plot

TrCKX2f1r1 amplification plot (A) and melting curve (B), qPCR performed with leaf cDNAs and the *TrCKX2* primers TrCKX2F1/TrCKX2R1.

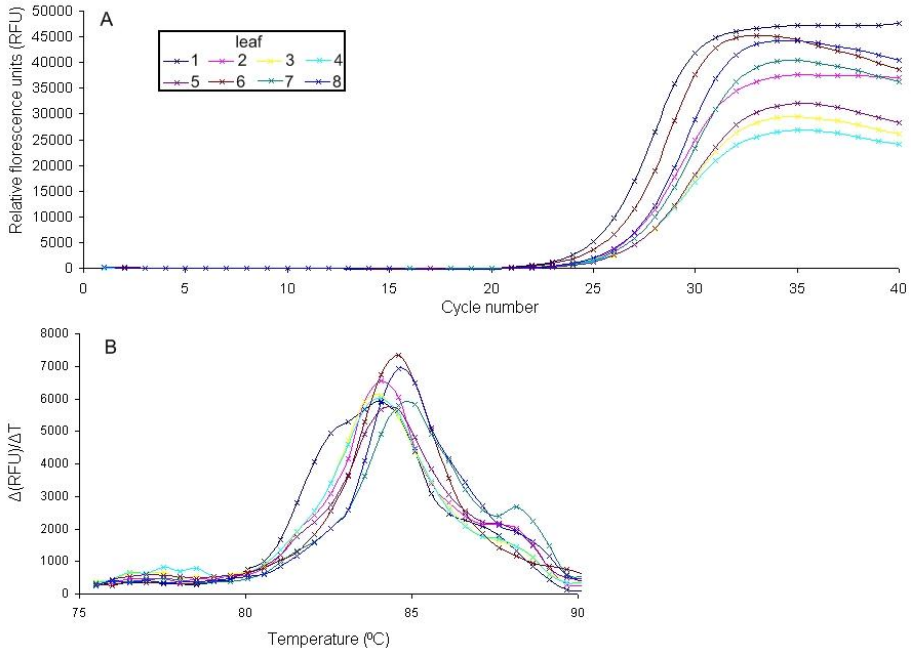


Figure 4.17 *TrCKX6* amplification plot

TrCKX6 amplification plot (A) and melting curve (B), qPCR performed with leaf cDNAs and the *TrCKX6* primers TrCKX6F1/TrCKX6R2.

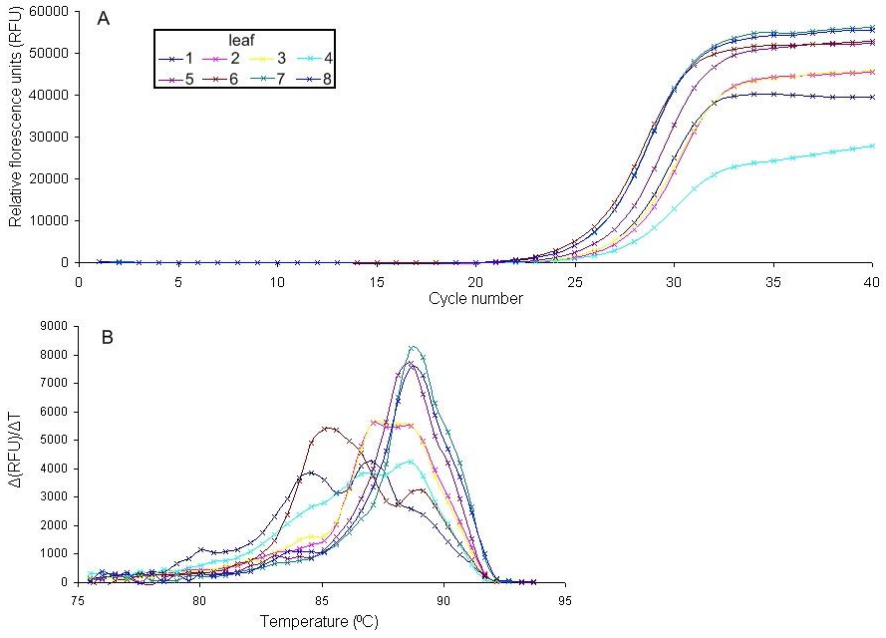


Figure 4.18 *TrCKX7* amplification plot

TrCKX7 amplification plot (A) and melting curve (B), qPCR performed with leaf cDNAs and the *TrCKX7* primers TrCKX7F2/TrCKX7R1.

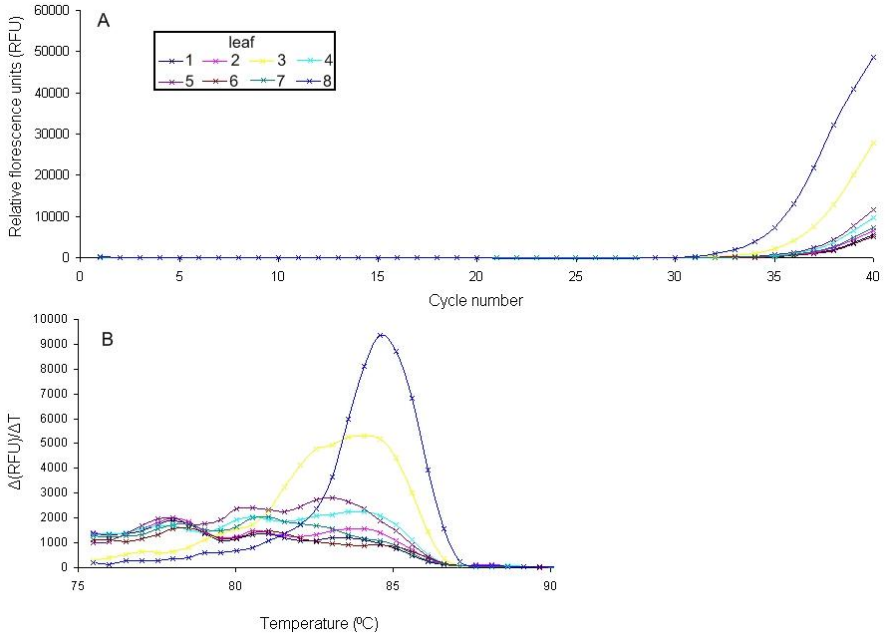


Figure 4.19 *TrIPT1* amplification plot

TrIPT1 amplification plot (A) and melting curve (B), qPCR performed with leaf cDNAs and the *TrIPT1* primers TrIPT1F1/TrIPT1R1.

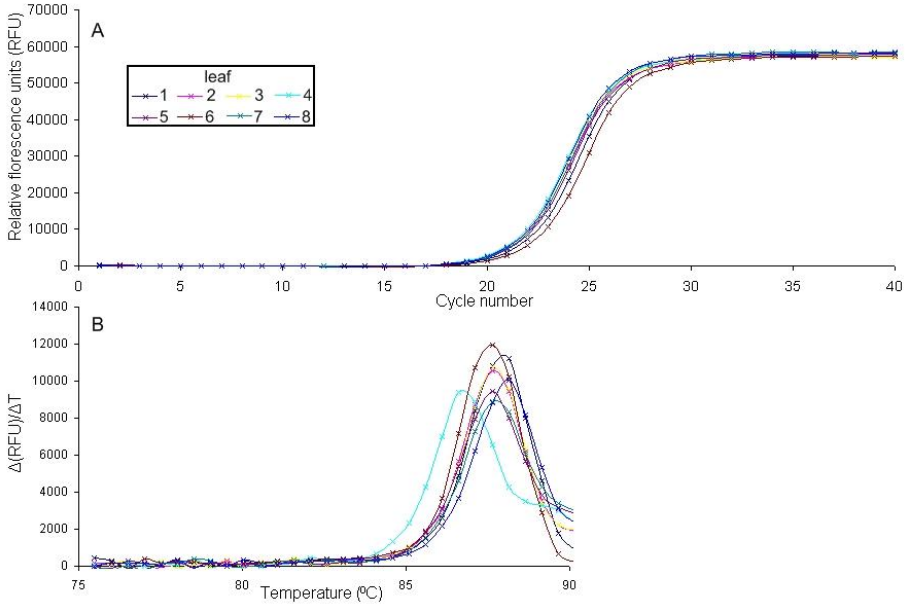


Figure 4.20 *TrCKX1* amplification plot

TrCKX1 amplification plot (A) and melting curve (B), qPCR performed with leaf cDNAs and the *TrCKX1* primers TrCKX1F1/Tr CKX1R1.

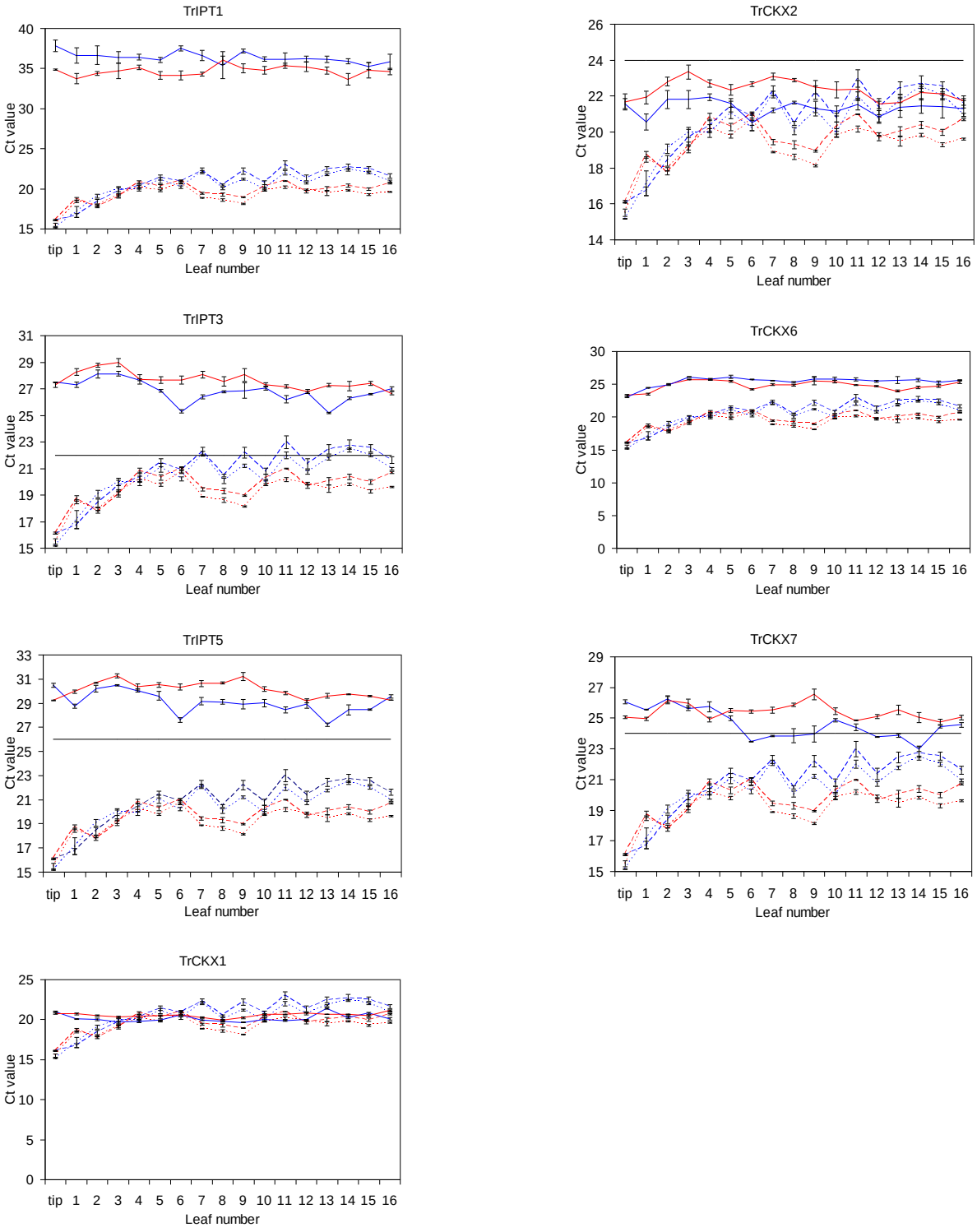


Figure 4.21 Average Ct values through development

Threshold cycle values (Ct) for *TrIPT1*, *TrIPT3*, *TrIPT5*, *TrCKX1*, *TrCKX2*, *TrCKX6* and *TrCKX7* plotted alongside *ACT* (---) and *GAP* (····) for the developmental leaf series from two plants plant 5 (blue) and plant 6 (red) The horizontal line in *TrIPT3*, *TrIPT5*, *TrCKX2* and *TrCKX7* represents the detection limit as determined by template dilution . Results are mean Ct values ± SE; n = 3.

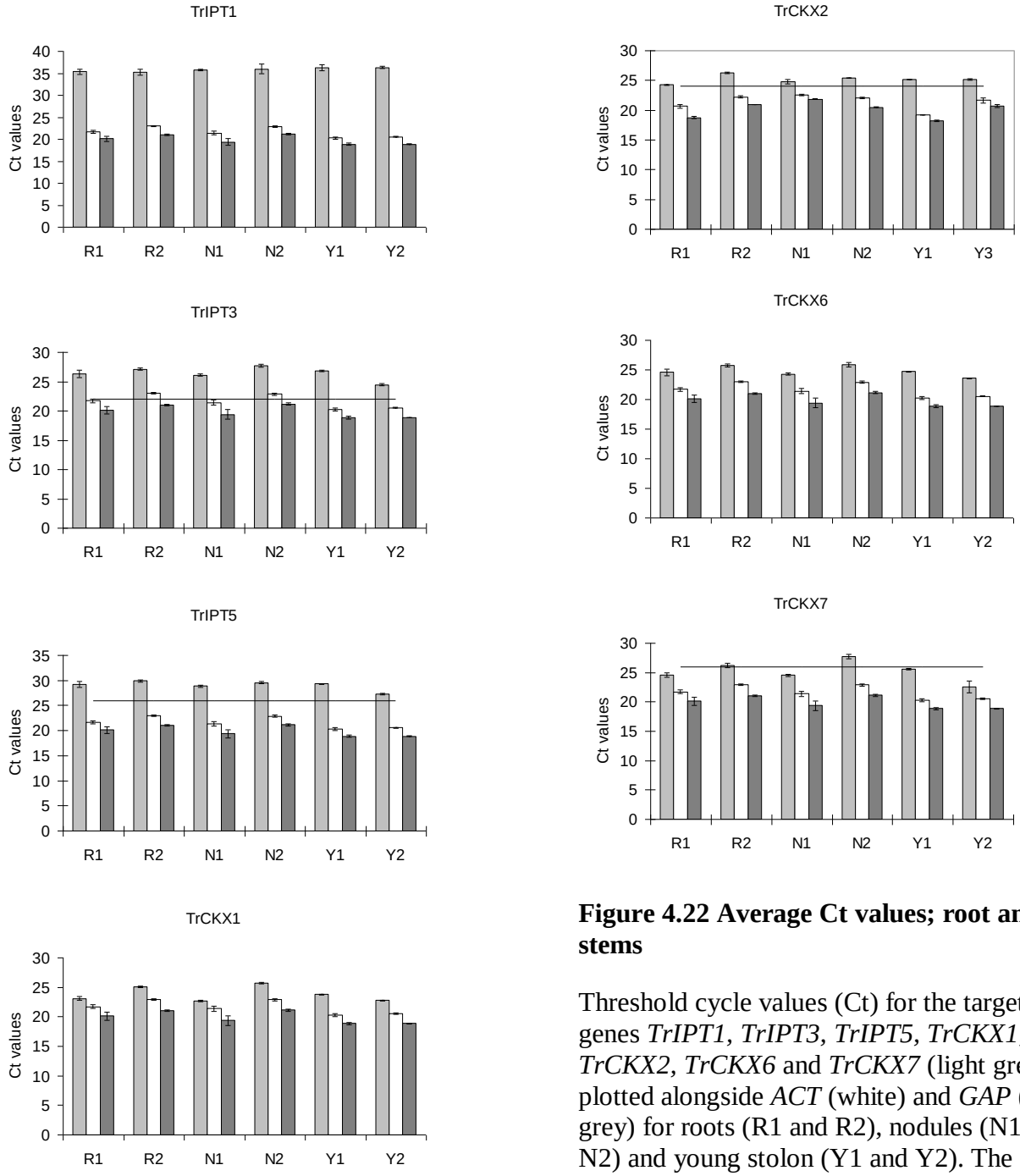


Figure 4.22 Average Ct values; root and stems

Threshold cycle values (Ct) for the target genes *TrIPT1, TrIPT3, TrIPT5, TrCKX1, TrCKX2, TrCKX6* and *TrCKX7* (light grey) plotted alongside *ACT* (white) and *GAP* (dark grey) for roots (R1 and R2), nodules (N1 and N2) and young stolon (Y1 and Y2). The horizontal line in *TrIPT3, TrIPT5, TrCKX2* and *TrCKX7* represents the detection limit as determined by template dilution. Results are mean Ct values ± SE; n = 3.

For the house keeping genes, *ACT* and *GAP*, the first 3-4 tenfold dilutions of the template moved the amplification curve by the expected ~3.3 cycles (Figure 4.23 and Figure 4.24) and the melting curves produced single peaks at the expected 85°C and 86°C for ACT and GAP, respectively. Thereafter, all further dilutions resulted in amplification curves that emerged in the same point. This was accompanied by corresponding degeneration of the melting curves (Figure 4.23 and Figure 4.24). Gel electrophoresis of the qPCR products show that at high template concentration both ACT and GAP reactions produced a single product at the expected size (

Figure 4.25). As the template concentration decreased the concentration of the specific product decreased alongside the emergence of a non-specific product. The ACT qPCR assay provided an accurate measurement of the template concentration up to Ct ≈ 22 and the GAP qPCR assay provided an accurate measurement up to Ct ≈ 25. Beyond this, dilutions failed to produce the expected 3.3 cycle increase in Ct values.

The first few 10-fold template dilution cycles produced the expected 3.3 cycle shifts in amplification plots for the *IPT* and *CKX* genes (three cycles for *TrCKX7* (Figure 4.26), four cycles for *TrIPT3* (Figure 4.27) and *TrCKX2f1r1* (Figure 4.28), and five cycles for *TrIPT5* (Figure 4.29)). qPCR reactions were insensitive to all following template dilutions, with all the amplification curves emerging at the same point. The melting curves also showed a distinct change with template concentration. The melting curves from *TrIPT3* had two peaks at high template concentrations with primary peaks at 82°C and 83°C respectively. At lower template concentrations the primary peak was reduced and the secondary peaks became dominant. The melting curve peaks for the other genes were broader and less sharp at lower template concentrations.

Gel electrophoresis of the PCR products showed that through the dynamic range, where Ct values increase in response to template dilution, a specific product was produced at the expected size. When the template concentration fell below the detection limit no specific product was produced and only non-specific products were produced (Figure 4.30)

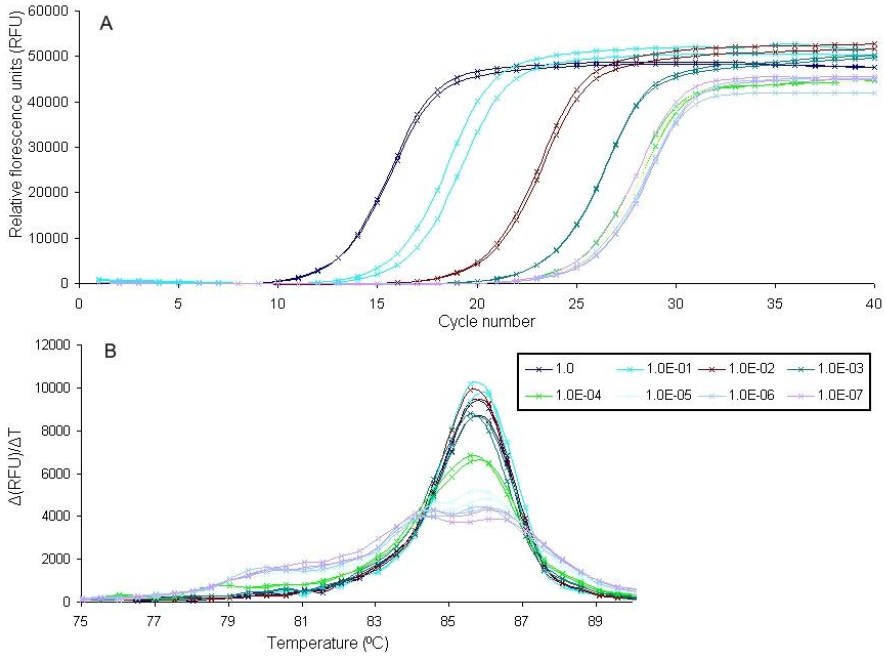


Figure 4.23 ACT serial dilution

ACT amplification plot (A) and melting curve (B). qPCR performed with a serial dilution of purified *ACT* PCR product as the template and the primers TrACTF2/ACTR2.

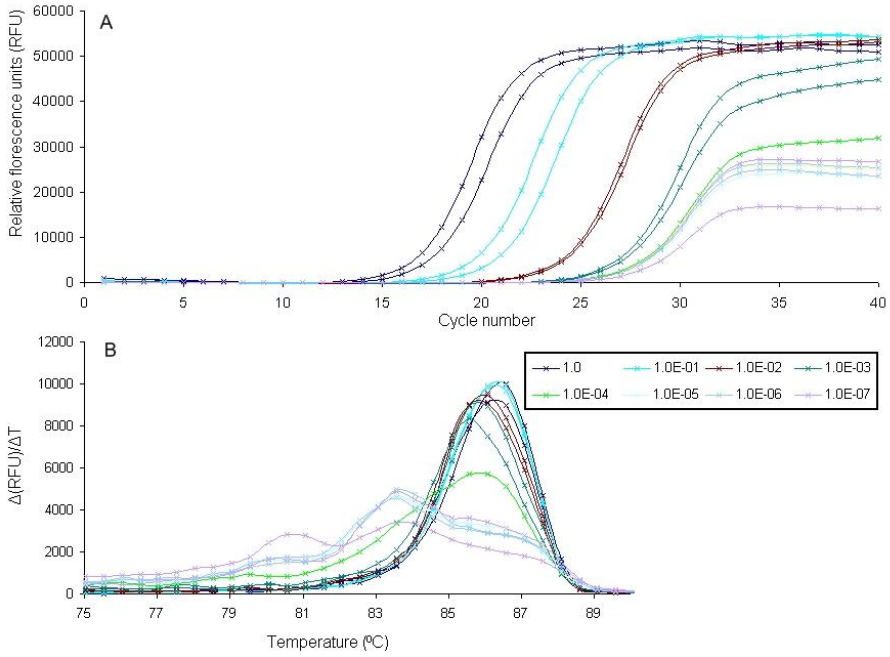


Figure 4.24 GAP serial dilution

GAP amplification plot (A) and melting curve (B). qPCR performed with a serial dilution of purified *GAP* PCR product as the template and the primers TrGAPF2/TrGAPR2.

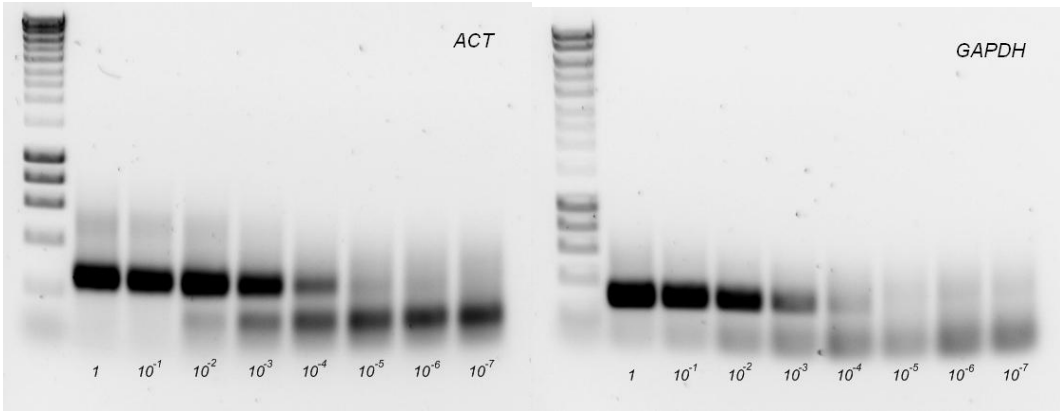


Figure 4.25 PCR products from ACT and GAP serial dilutions

Agarose gels showing the qPCR product produced for *ACT* and *GAP* for a series of dilutions of gene specific DNA templates. Numbers represent relative template concentrations.

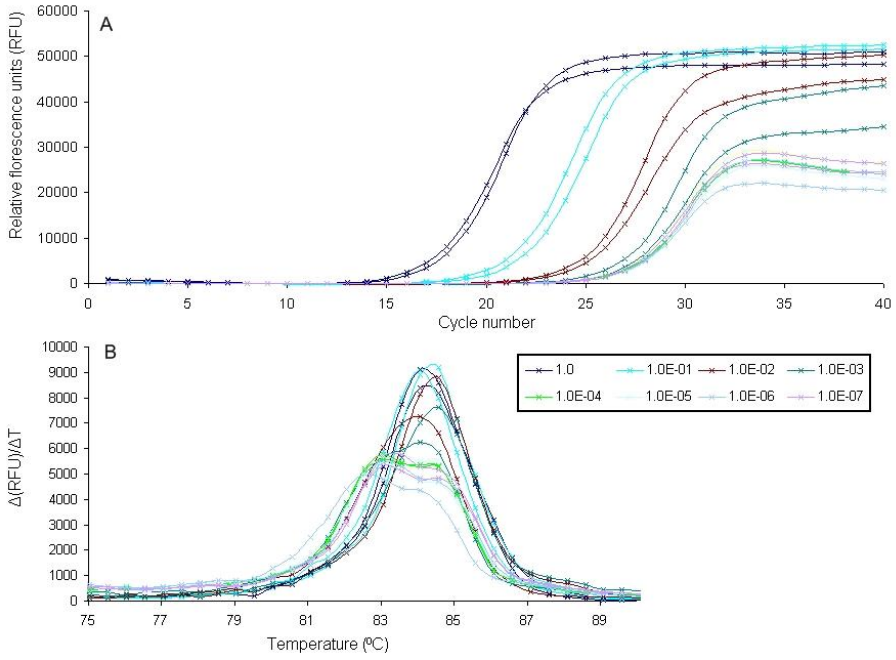


Figure 4.26 *TrCKX7* serial dilution

TrCKX7 amplification plot (A) and melting curve (B), qPCR performed with a serial dilution of purified *TrCKX7* PCR product as the template and the primers TrCKX7F2/TrCKX7R1.

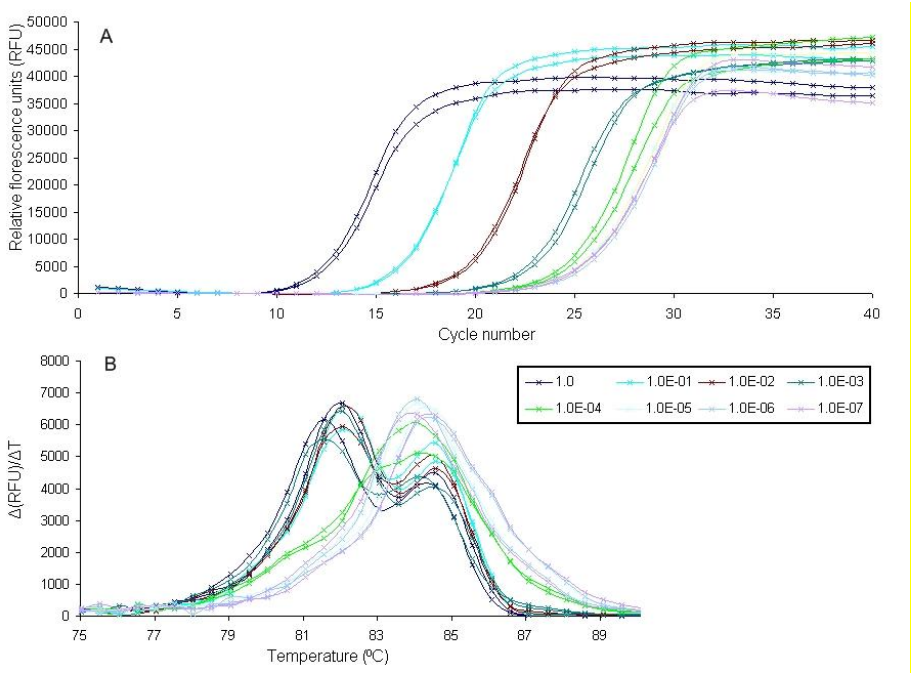


Figure 4.27 *TrIPT3* serial dilution

TrIPT3 amplification plot (A) and melting curve (B), qPCR performed with a serial dilution of purified *TrIPT3* PCR product as the template and the primers TrIPT3F1/TrIPT3R1.

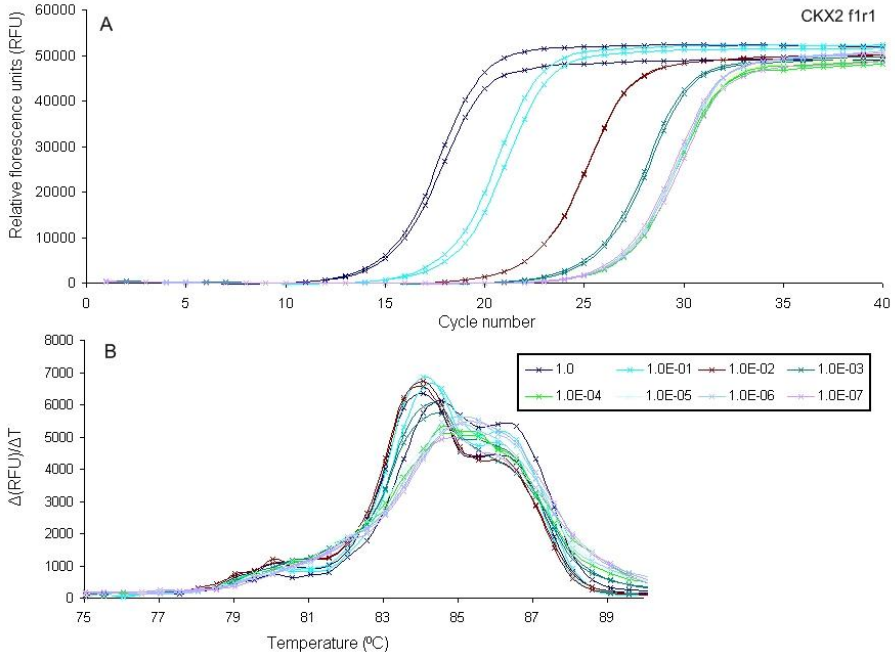


Figure 4.28 *TrCKX2* serial dilution

TrCKX2 amplification plot (A) and melting curve (B), qPCR performed with a serial dilution of purified *TrCKX2* PCR product as the template and the primers TrCKX2F1/TrCKX2R1.

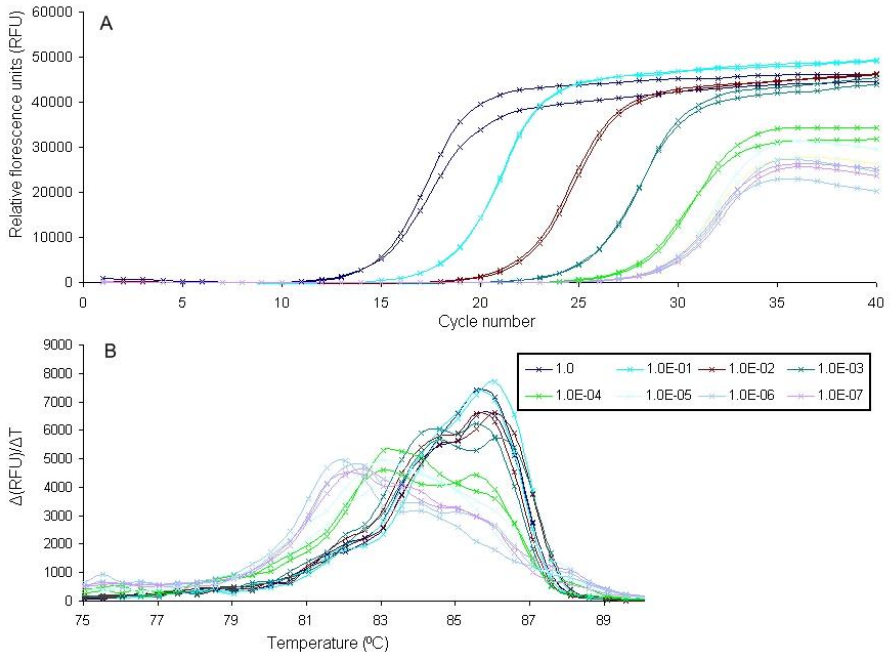


Figure 4.29 *TrIPT5* serial dilution

TrIPT5 amplification plot (A) and melting curve (B), qPCR performed with a serial dilution of purified *TrIPT5* PCR product as the template and the primers TrIPT5F2/TrIPT5R2.

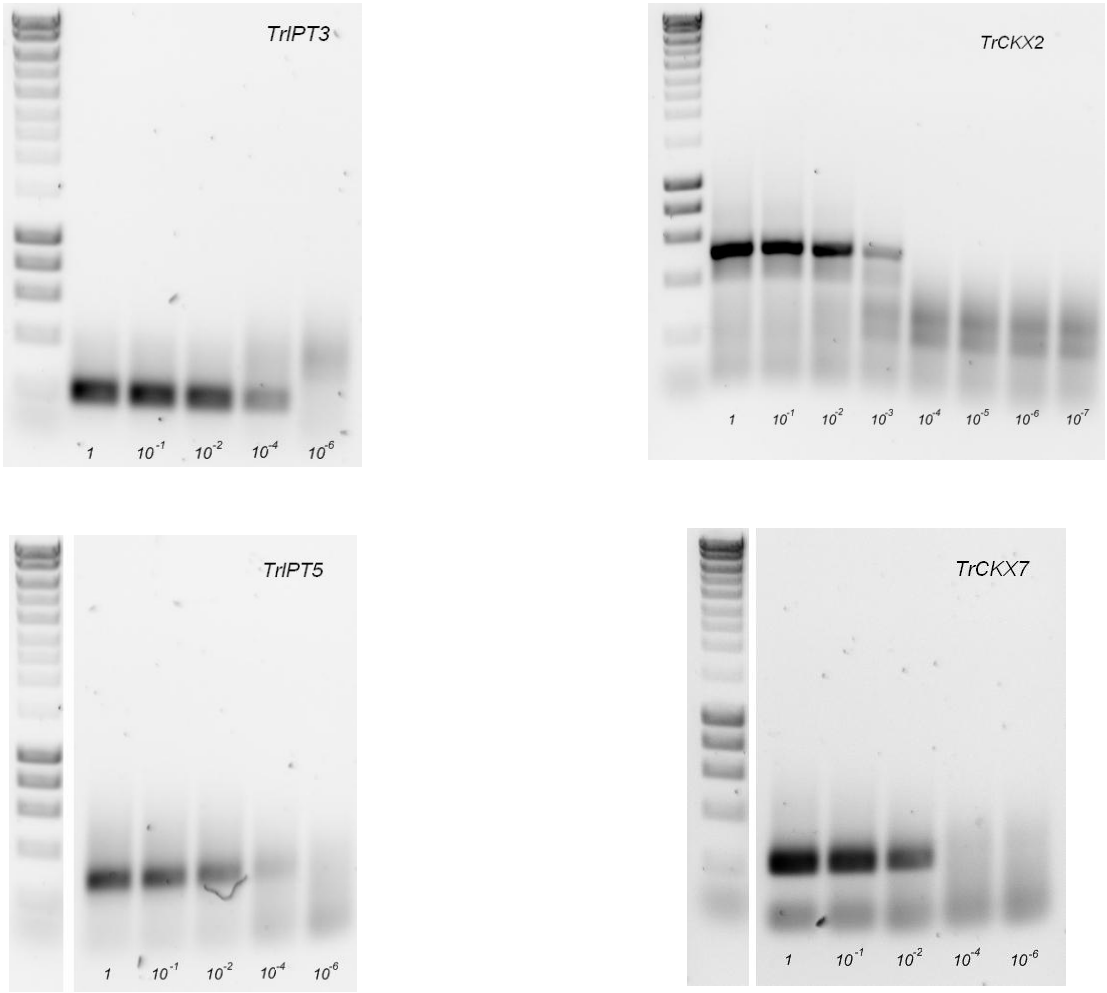


Figure 4.30 Agarose gels for *TrIPT3, TrIPT5, TrCKX2* and *TrCKX7 dilution series*

Agarose gels showing the PCR product produced with qPCR for *TrIPT3*, *TrIPT5*, *TrCKX2* and *TrCKX7* with 10-fold dilutions of gene specific DNA templates, numbers represent relative template concentrations.

Variations in gene expression are only detectable when transcript levels are relatively high, based on the dilution series data. The detection limits for tested genes are as follows: *TrIPT3* < 22, *TrIPT5* <26, *TrCKX2* < 24, *TrCKX7* < 24, *GAP* < 25, *ACT* < 22. The sensitivity of the *TrIPT1* assay was not defined by template dilution but produced sharp defined melting peaks when Ct values were less than 34; only a faint product is detected in the qPCR product (Figure 4.31). *TrCKX1* lacks specificity and produced multiple non specific products with some cDNAs (Figure 4.31).

From this the qPCR assay has been shown to only reliably detect the expression of the house keeping genes *ACT, GAP* and *TrCKX2*. Expression of TrCKX7 was close to the detection limit. Expression of all the other genes was below the detection and or lacked specificity

4.3.2 Quantitative gene expression

Quantification of the relative gene expression was determined for *TrCKX2* and *TrCKX7*. Expression of both *TrCKX2* and *TrCKX7* was lowest in the apical tip and generally increased through leaf development (Figure 4.32 and Figure 4.33). *TrCKX2* expression was highest in mature and senescence leaves (Figure 4.32). Although *TrCKX2* expression generally increased with leaf age there were considerable fluctuation between adjacent leaves and little correlation between biological replicates. Plant 5 expression was consistently higher in all leaves except for the apical tip and leaf 1. This aside *TrCKX2* expression was most stable in senescent leaves (leaf 11 onwards). Expression of *TrCKX7* is considerably lower in the apical tip than all other tissues where expression is fairly constant (Figure 4.33). In leaves, *TrCKX7* expression increased thorough out leaf expansion reaching a plateau around the fifth leaf and thereafter remaining fairly constant. Plant 5 again showed consistently higher expression. Plant 5 also lacked the dip in expression between leaves 7 to 9 as observed in plant 6. Given that much of the *TrCKX7* expression data was below the estimated limit of detection (Figure 4.21 and Figure 4.22) the patterns observed may be an artifact of the experiment.

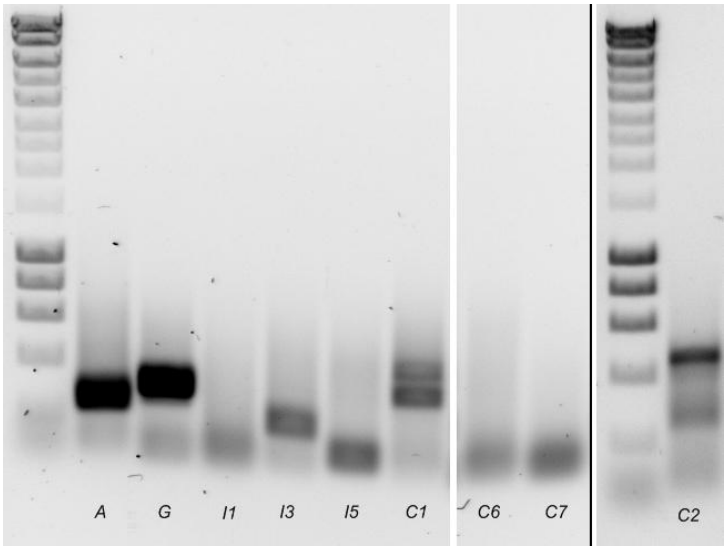
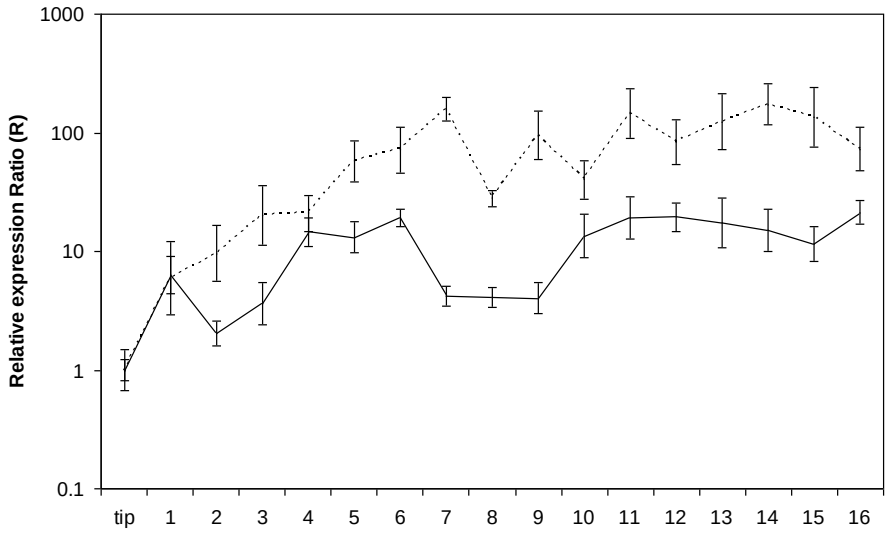


Figure 4.31 Gel of all qPCR products

qPCR products for *ACT (A)*, *GAP (G)*, *TrIPT1 (I1)*, *TrIPT3 (I3)*, *TrIPT5 (I5)*, *TrCKX1 (C1)*, *TrCKX6 (C6)*, *TrCKX7 (C7)* and *TrCKX2 (C2)*. PCR products of the expected size are produced for *ACT* (240 bp), *GAP* (296 bp), *TrIPT3* (190 bp) and *TrCKX2* (551 bp). A faint product is present for *TrIPT1* (150 bp). Multiple products were produced by *TrCKX1*. No clearly defined product was formed in *TrIPT5*, *TrCKX6* and *TrCKX7* at the expected sizes of 320 bp, 206 bp and 290 bp respectively. Results for *TrCKX2* show leaf 7, all others for nodule 2.

A



B

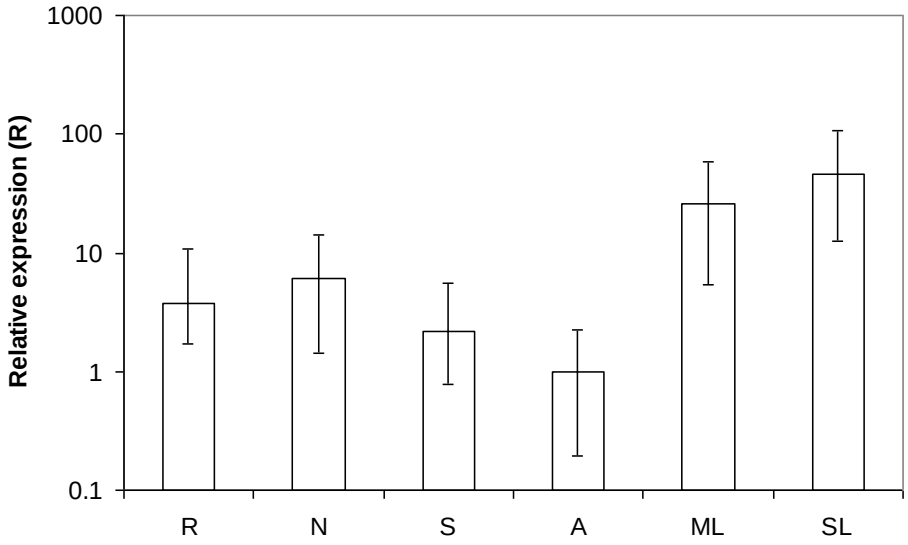
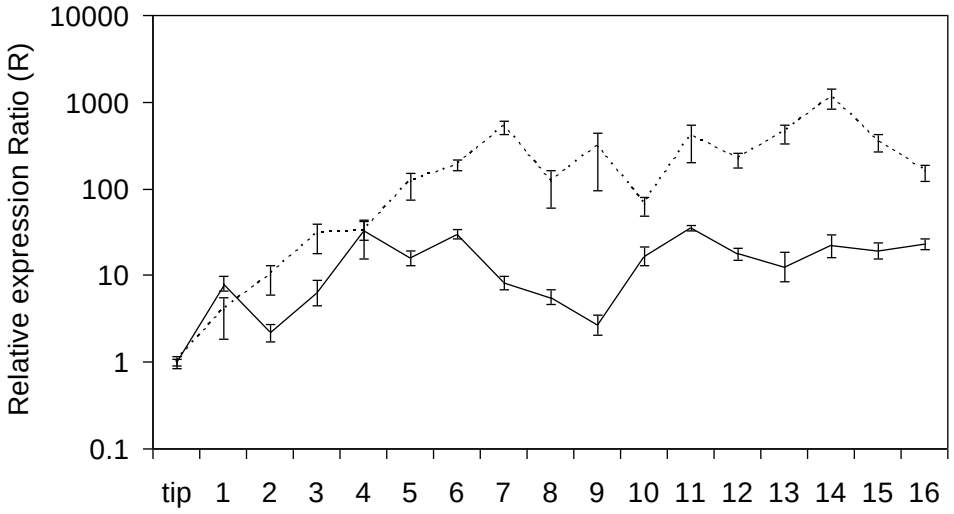


Figure 4.32 Relative expression of *TrCKX2*

Expression of *TrCKX2* relative to the house keeping genes *ACT* and *GAP*, plotted on a log scale, through leaf development (**A**) and across the plant (**B**). **A:** Plant 5 shown as a dotted line and plant 6 as a solid line, Results are mean values ± SE; n = 3. **B:** expression in roots (R), nodules (N), stolon (S), apical tip (A), mature leaves (ML) and senescent leaves (SL). Relative expression is plotted on a log scale. Results are mean values ± SE; 2 biological replicates with 3 technical replicates for R, N, S and A, ML and SL are pooled results leaves 4 to 6 and 11 to 13 respectively, with 2 biological replicates and 3 technical replicates per leaf.

A



B

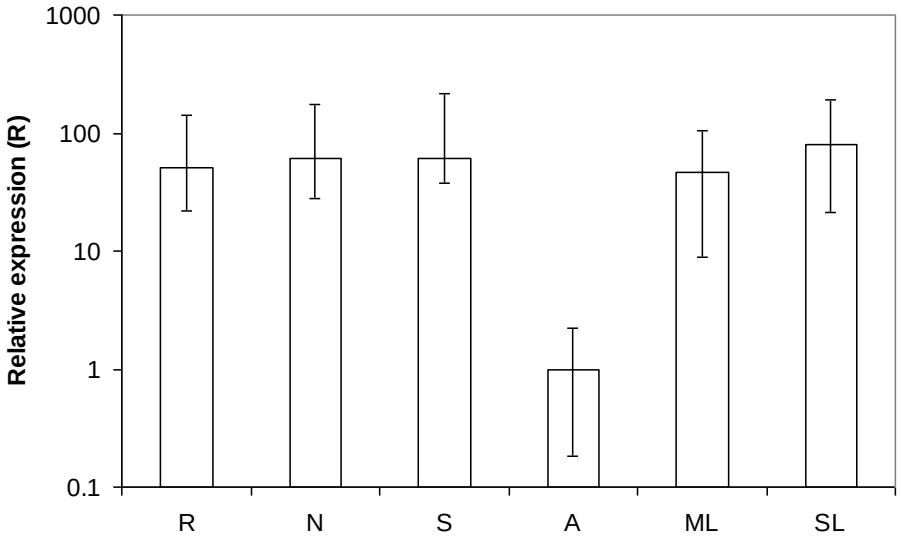


Figure 4.33 Relative expression of *TrCKX7*

Expression of *TrCKX7* Relative to the house keeping genes *ACT* and *GAP* through leaf development (**A**) and across the Plant (**B**). **A;** Plant 5 shown as a dotted line and plant 6 as a solid line, Results are mean values ± SE; n = 3. **B;** expression in roots (R), nodules (N), stolon (S), apical tip (A), mature leaves (ML) and senescent leaves (SL). Relative expression is plotted on a log scale. Results are mean values ± SE; 2 biological replicates with 3 technical replicates for R, N, S and A, ML and SL are pooled results leaves 4 to6 and 11 to 13 respectively, with 2 biological replicates and 3 technical replicates per leaf.

Chapter 5

Discussion

5.1 NanoDrop Chlorophyll determination

Chlorophyll assays performed with traditional spectrophotometers require a comparatively large sample volume, and have comparatively narrow effective absorbance range which necessitates the dilution of samples to bring them into range. The NanoDrop requires only 2 µl of sample allowing very small samples to be analysed and with equivalent absorbance range of up to 65 absorbance units, sample dilutions are largely eliminated. Although the Novaspec was more accurate than the NanoDrop, NanoDrop the provided a fast and efficient means of measuring relative chlorophyll from very small leaf samples.

5.2 Gene identification and characterisation

The monocot and dicot IPT gene families appear to have diverged after the monocot dicot split (Figure 4.9) (Kakimoto, 2001; Sakamoto et al., 2006). Amongst the dicot genes there are two clades, one containing *AtIPT1, AtIPT14, AtIPT16* along with *LjIPT1, TrIPT2* and *TrIPT5*, and a second clade containing *AtIPT3, AtIPT* 5, *AtIPT* 7 and most of the legume *IPT* genes including *TrIPT1, TrIPT3* and *TrIPT4*. Although the base of the phylogeny is not well resolved the results are consistent with Sakamoto (2006), Takei (2001) and Kakimoto (2001). *TrIPT5* is the only clover gene that did not associate with either of the two dicot clades. Support for the placement of *TrIPT5* within any clade in the phylogeny is not high (Figure 4.9). Only a short 3' fragment of the *TrIPT5* was identified which provided only limited data for the tree building algorithms. *TrIPT5* was found to be transcribed in nodes and leaves. Further study of this gene including isolation of the full length coding sequence is required.

The clover gene *TrIPT1* is homologous to *LjIPT3* (Figure 4.9). Low level expression of *LjIPT3* has been detected in nodule EST data bases (UniGene Lja.12109). *TrIPT1* was found to be expressed in a range of clover tissues including senescent leaves. Aside from any role in leaf development the similarity of *TrIPT1* to *LjIPT3* implies a possible role in root nodulation.

We identified a full length coding sequence for *TrIPT2* from the pastoral genomics database. RT-PCR with *TrIPT2* specific primers failed to isolate *TrIPT2* transcripts in any of the cDNAs tested. As such we can not confirm that this gene is transcribed in clover. TrIPT2 has a high degree of homology to *LjIPT1* (Figure 4.9). Little is known of the expression of the *Lotus japonicas* gene *LjIPT1*. *TrIPT2* is the only clover *IPT* from the second dicot *IPT* clade. The *Arabidopsis* members of this clade (*AtIPT1*, *AtIPT14*, *AtIPT16*) are expressed in a range of tissues from leaves, axillary buds, roots, floral tissues, fruit and developing seeds (Miyawaki et al., 2004). Further studies are required to determine the function of *TrIPT2*.

TrIPT4 and *TrIPT3* are present in a clade of legume *IPT* genes including *PsIPT1*, *PsIPT2*, *LjIPT2*, *LjIPT4* and *GmIPT* (Figure 4.9). Expression of the pea *IPT* genes, *PsIPT1* and *PsIPT2*, has been associated with the breaking of apical dominance in lateral buds (Tanaka et al., 2006). *TrIPT4* and to a lesser extent *TrIPT3* share homology with *PsIPT1* and *PsIPT2* which raises the possibility that *TrIPT4* and *TrIPT3* may play a similar role in controlling lateral bud release in white clover. We isolated *TrIPT3* transcripts from senescent leaves but did not find any evidence of *TrIPT4* expression. However none of the cDNAs used for gene identification were enriched for lateral bud specific genes. RT-PCR with a subtractive cDNA enriched for lateral bud specific genes as used by (Tanaka et al., 2006) may assist in isolation of *TrIPT4* transcripts. Traditionally cytokinins were thought to originate in the roots. In peas, lateral bud, dormancy is released by cytokinins produced by the local expression of *IPT* in the stem (Tanaka et al., 2006). Thomas et al. (2003) showed in clover that root development in proximal nodes encourages lateral shoot growth, implicating root sourced cytokinin in the release of lateral buds. How lateral buds are released in white clover and the role of root and locally produced cytokinin is yet to be resolved. Further studies of *TrIPT3* and *TrIPT4* expression may provide insights into the mechanisms regulating lateral bud growth in clover.

Unlike the *IPT* genes, *CKX* genes are more conserved and there is no obvious division between the monocot and dicot clades, implying a more ancient diversification within the *CKX* genes. Although bootstrap support values for our *CKX* phylogeny are not high, our phylogenetic tree (Figure 4.10) compares favourably with the results of Schmülling *et al.* (2003) and Ashikari *et al.* (2005).

TrCKX1 showed good homology to *PsCKX1* and the *Arabidopsis* gene *AtCKX7* (Figure 4.10). Very little is known about the expression of this group of genes. Both *AtCKX7* and *PsCKX1* have shown

relatively stable patterns of expression in qPCR studies (Vaseva-Gemisheva et al., 2005; Werner et al., 2006). We isolated and sequenced a fragment of *TrCKX1* from leaf and node cDNAs showing expression in these tissues. Further expression analysis is required to determine the function of *TrCKX1*.

TrCKX2 appears to be a member of a legume specific clade of *CKX* genes (Figure 4.10). BLAST and maximum parsimony analysis did not associate *TrCKX2* with any specific annotated *CKX* genes, although BLAST identified a number of homologous legume ESTs. Further studies are needed to investigate the nature of this legume specific clade. *TrCKX2* fragments were isolated and sequenced from senescent leaves showing expression in this tissue.

The three putative genes *TrCKX3*, *TrCKX4*, and *TrCKX5* form a clade with *AtCKX3* (Figure 4.10). This clade has low boot strap support although BLAST analysis shows close homology between these genes. There is no common sequence data available for *TrCKX4* and either *TrCKX3* or *TrCKX5*, with *TrCKX4* coding for a portion of the 3' end of a transcript and *TrCKX3* and *TrCKX5* coding for a 5' region. *TrCKX4* may not be a separate gene but code for the 3' region of either *TrCKX3* or *TrCKX5*. We did not detect expression of *TrCKX3*, *TrCKX4*, and *TrCKX5* in any of the cDNAs tested. The *Arabidopsi*s homologue, *AtCKX3*, is only expressed in a limited number of unidentified cell types (Werner et al., 2006) and is sparsely represented in EST databases. With this in mind it is likely that *TrCKX3*, *TrCKX4*, and *TrCKX5* expression is also limited to a small number of cell types. All the cDNAs used in this study were developed from whole tissues samples such as whole leaves or roots. If these genes are only expressed in a small sub set of cells in these samples, their proportion of total cDNAs may be below the detection limit.

The relationship between *TrCKX6* and the other *CKX* genes is not well defined. Phylogenetic and BLAST analysis of *TrCKX6* did not place it within any specific clade (Figure 4.10). The *TrCKX6* gene fragment codes for a conserved region of the gene, making design of specific primers for *TrCKX6* problematic. Sequencing of the complete coding sequence may be necessary before specific *TrCKX6* primers can be developed.

TrCKX7 shows homology to the *Arabidopsis* genes *AtCKX1* and *AtCKX6* (Figure 4.10). *AtCKX1* is expressed in floral tissues, vegetative apex and root branching points (Werner et al., 2003) and *AtCKX6* is expressed in the vascular and floral tissues (Werner et al., 2003; Carabelli et al., 2007).

In the *Arabidopsis* shade avoidance response, suppression of growth in the primordium leaf is achieved by reduction in cytokinin via the auxin induced expression of *AtCKX6* (Carabelli et al., 2007). We identified *TrCKX7* transcription in expanded and senescent leaves. If *TrCKX7* plays a similar role to AtCKX6 in controlling leaf development this gene may provide insights to the regulation of leaf development in clover. Both *AtCKX1* and *AtCKX6* are expressed in floral tissues (Werner et al., 2003). Over expression of *AtCKX1* increases seed size (Werner et al., 2003). The similarity of *TrCKX7* to these genes may be of interest for those people studying clover seed development.

The experimental plants were found to be infected with the white clover mosaic virus. Clark et al. (1999) found that infection of beans (*Phaseolus vulgaris*) with the white clover mosaic virus affected the composition of cytokinin species in infected leaves. What effect the virus infection has on the expression of *IPT* and *CKX* genes and the validity of our results is unknown. Further analysis of the expression of these genes in virus infected plants may provide insights into the plant responses to viral infection.

5.3 CKX2 and CKX7 expression

The expression of *TrCKX2* during leaf development was the only gene where expression was above the limit of detection of the qPCR assay. The expression of *TrIPT2* in roots, nodules and stolons and *TrCKX7* expression in most tissues were at the limit of detection. The method used to determine the reaction sensitivity of the qPCR assay utilised serial dilutions of purified PCR product as the DNA template whereas the gene expression study used cDNA. In cDNA the concentration of the target gene may be small, although the nucleotide concentration is comparatively high. Many of the reactions performed with cDNA produced Ct values larger than those produced with the diluted PCR product. This implies that the two reactions are not directly comparable and that reactions performed with cDNA may be more accurate than estimated. Given the uncertainty around the detection limit, the expression values determined for *TrCKX7* and the expression of *TrCKX2* in roots, nodules and stolons may be over estimated and should be treated with caution.

Cytokinins play an important role in senescence. Application of cytokinin delays senescence whether the cytokinin is applied externally or synthesised within the plant via the induction of the cytokinin synthesis enzyme *IPT*. Further to this, senescence can be delayed by the synthesis of

cytokinin within the senescent tissue (Smart et al., 1991; Li et al., 1992; Hewelt et al., 1994; Gan and Amasino, 1995; Rivero et al., 2007) or by cytokinin synthesised elsewhere within the plant (McKenzie et al., 1998). The progression of senescence appears to be reliant on reduction of active cytokinin within the tissue. In barley, *CKX* has been shown to be up regulated in senescing detached barley leaves (Conrad et al., 2007). We hypothesised that senescence in white clover may be regulated by a reduction in leaf cytokinin caused by the up regulation of *CKX* within the leaf.

The expression of *TrCKX2* and *TrCKX7* increases as leaves develop until around leaf 5 and thereafter expression was quite variable. The method used for the propagation of clover plants requires the removal of lateral shoots from the central stolon. The removal of apical buds in peas induces cytokinin production in the nodal stem (Tanaka et al., 2006). What effect the removal of lateral shoots has on the production of cytokinin in the associated nodes in clover and any effect this may have on the adjacent leaves is unknown. The variable *CKX* expression observed in mature leaves may be a response to the excision of nearby lateral shoots influencing the local cytokinin pool.

Although there was no profound change in gene expression at the onset of senescence *TrCKX2* and to a lesser extent *TrCKX7* were highly and more steadily expressed in senescent leaves than in mature leaves. From this result it does not appear that either *TrCKX2* or *TrCKX7* induce senescence. However the consistently high expression of these genes throughout senescence is consistent with results of Conrad et al.(2007), who found an increase in *CKX* activity in senescing barely leaves and proposed that *CKX* does not induce senescence but facilitates the progression of senescence.

5.4 Conclusions

This study consisted of two parts. Firstly, the development of an assay for the spectral determination of chlorophyll using the NanoDrop Spectrophotometer that allowed the determination of chlorophyll from part samples of clover leaves while leaving sufficient tissue for multiple RNA extractions. Secondly, the isolation of the cytokinin biosynthetic and metabolic genes *IPT* and *CKX* from white clover and investigation of their expression during developmental leaf senescence. Five putative *IPT* genes and seven putative *CKX* genes were identified. RT-PCR demonstrated the expression of seven of these genes (*TrIPT1*, *TrIPT3*, *TrIPT5*, *TrCKX1*, *TrCKX2*, *TrCKX6* and *TrCKX7*) and analysis with qPCR found a potential role for *CKX* in facilitating the progression of

development leaf senescence.

5.5 Future research directions

Further analysis of the expression of the *IPT* and *CKX* genes is required to determine what role these genes play in modulating cytokinin homeostasis throughout development in clover. Ideally qPCR primers should be developed for the 3' end of the gene as they are less affected by RNA degradation and incomplete cDNA synthesis. The design of our qPCR primers was complicated by a lack of sequence data with many of our gene fragments coding for the 5' end of the genes. Isolation of more complete gene sequences will allow for the design of more efficient and gene specific primers for qPCR. As *IPT* and *CKX* genes tend to be specifically and transiently expressed in small groups of cells, they may be at very low concentrations within cDNAs constructed from large samples. Micro dissection of leaves and other tissues may prove a useful technique for assaying the expression of such genes. Correcting expression with tissue specific housekeeping genes may further increase the resolution of such assays.

Other approaches would be to generate plants transformed with promoter:reporter gene constructs for the various *IPT* and *CKX* genes and/or a histological examination of plant tissues using in-situ hybridisation or probes for the specific *IPT* and *CKX* proteins. This would aid in the identification of the genes which are likely to be involved in regulating senescence and to define the locations within the tissues that cytokinins are produced and metabolised. This may improve our understanding of how cytokinin regulates the physiological processes of senescence.

Although several studies have shown endogenous cytokinin levels to decrease in leaves during senescence (Van Staden et al., 1988; Singh et al., 1992) as yet none have investigated this in clover. Measuring the concentration of the various cytokinin species throughout leaf development within leaves and associated tissues such as the petiole, nodes, stems and roots will prove useful in understanding the dynamics of cytokinin production, transport, modification and degradation.

Increases in the levels of cytokinin O-glucosides have been observed before the onset of senescence (Van Staden, 1996; Taverner et al., 1999). It has been proposed that O-glycosylation of active cytokinins may be responsible for reducing the active cytokinin pool in senescent tissues thereby facilitating the development of senescence in some plants this may be the process which controls

developmental senescence in white clover. At present no cytokinin O-glucosyltransferase genes have been isolated from white clover, although a number cytokinin O-glucosyltransferases have been isolated from other legumes. Further studies of the expression of white clover cytokinin O-glucosyltransferase during leaf development will enhance our understanding the role of O-glycosylation in senescence.

Chapter 6

Appendix

6.1 sequences

6.1.1 *Trifolium repens* isopentenyltransferases genes

Gene	Species	Identifier code	Code type
TrIPT1	***Trifolium repens***	**unpublished**	
TrIPT2	***Trifolium repens***	**CTR0036009640-cF16_20040726**	**PG Subject id**
TrIPT2	***Trifolium repens***	**CTR0036049166-cF3_20040726**	**PG Subject id**
TrIPT2	***Trifolium repens***	**CTR0036049166-cF3_20040726**	**PG Subject id**
TrIPT3	***Trifolium repens***	**FTRC101567L23-b0FSP_20030715**	**PG Subject id**
TRIPT4	***Trifolium repens***	**CTR0036032609-cF7_20040726**	**PG Subject id**
TrIPT5	***Trifolium repens***	**CTR0036033096-cF2_20040726**	**PG Subject id**

6.1.2 *Trifolium repens* cytokinin-oxidase/dehydrogenase genes

Gene	Species	Identifier code	Code type
TrCKX1	***Trifolium repens***	**EF691439**	**PG Subject id**
TrCKX2	***Trifolium repens***	**CTR0036048420-cF2_20040726**	**PG Subject id**
TrCKX3	***Trifolium repens***	**CTR0036060683-cF2_20040726**	**PG Subject id**
TrCKX4	***Trifolium repens***	**CTR0036082477-cF2_20040726**	**PG Subject id**
TrCKX5	***Trifolium repens***	**FTRC101608C21-g0RSP_20030715**	**PG Subject id**

TrCKX6	***Trifolium repens***	**FTRC101536F05-b0FSP_20030602**	**PG Subject id**
TrCKX7	***Trifolium repens***	**CTR0036064318-cF2_20040726**	**PG Subject id**

6.1.3 Sequences used for developing TDipt4 F1 and TDipt4 R1

Species	Identifier code	Code type
Medicago truncatula	***AC146865***	**Accession No**
Lotus japonicus	***DQ436464***	**Accession No**
Arabidopsis thaliana	***NM_116176***	**Accession No**
Vitis vinifera	***AM483252***	**Accession No**

6.1.4 Sequences used for developing TDckx2F1 and TDckx2R1 primers

species	Identifier code	Code type
Trifolium repens	**EF691439**	**Accession No**
Glycine max	**TC232674**	**TIGR TC Identifier**
Medicago truncatula	**TC95407**	**TIGR TC Identifier**
Pisum sativum	**EF030477**	**Accession No**

6.1.5 Genes used for developing housekeeping primers for GAP, ACT and PP2

Gene	Species	Identifier code	Code type
ACT	***Trifolium repens***	**CTR0036061012-cF6_20040726**	**PG Subject id**
	Trifolium pratense	**AY372368**	**Accession No**
	Trifolium repens	**AM419900**	**Accession No**
GAP	***Trifolium repens***	**CTR0036085039-cF6_20040726**	**PG Subject id**
PP2	***Medicago sativa***	**AF196286**	**Accession No**
	Pisum sativum	**Z25888**	**Accession No**
	Trifolium repens	**CTR0036042845-cF2**	**PG Subject id**
	Trifolium repens	**CTR0036053123-cF5_20040726**	**PG Subject id**
	Trifolium repens	**CTR0036059084-cF10_20040726**	**PG Subject id**
	Trifolium repens	**CTR0036059085-cF2_20040726**	**PG Subject id**
	Trifolium repens	**CTR0036078442-cF2_20040726**	**PG Subject id**
	Trifolium repens	**CTR0036081315-cF3_20040726**	**PG Subject id**
	Trifolium repens	**CTR0036081316-cF2_20040726**	**PG Subject id**
	Trifolium repens	**ETOSTORX15A07-g1M13RE**	**PG Subject id**
	Trifolium repens	**ETRN16RX12E03-g1M13RE_20030618**	**PG Subject id**
	Trifolium repens	**FTRC101090E04-g0RSP_20021213**	**PG Subject id**
	Trifolium repens	**TR0036053125-cF3_20040726**	**PG Subject id**

6.1.6 *IPT* genes used for bioinformatics

Gene name	Species	Accession No
AtIPT1	***Arabidopsis thaliana***	**AB062607.1**
AtIPT3	***Arabidopsis thaliana***	**BT001075.1**
AtIPT4	***Arabidopsis thaliana***	**AB062611.1**
AtIPT5	***Arabidopsis thaliana***	**AB062608.1**
AtIPT6	***Arabidopsis thaliana***	**AB062612.1**
AtIPT7	***Arabidopsis thaliana***	**AB062613.1**
AtIPT8	***Arabidopsis thaliana***	**AB062614.1**
LjIPT1	***Lotus japonicus***	**DQ436462**
LjIPT2	***Lotus japonicus***	**DQ436463**
LjIPT3	***Lotus japonicus***	**DQ436464**
LjIPT4	***Lotus japonicus***	**DQ436465**
PsIPT1	***Pisum sativum***	**AB194606**
PsIPT2	***Pisum sativum***	**AB194607**
GmIPT1	***Glycine max***	**AY550884**
ZmIPT1	***Zea mays***	**EU263125**
ZmIPT2	***Zea mays***	**EU263126**
ZmIPT4	***Zea mays***	**EU263127**
ZmIPT5	***Zea mays***	**EU263128**
ZmIPT6	***Zea mays***	**EU263129**
ZmIPT7	***Zea mays***	**EU263130**
ZmIPT8	***Zea mays***	**EU263131**
OsIPT1	***Oryza sativa***	**AB239797**
OsIPT2	***Oryza sativa***	**AB239798**
OsIPT3	***Oryza sativa***	**AB239799**
OsIPT4	***Oryza sativa***	**AB239800**
OsIPT5	***Oryza sativa***	**AB239801**
OsIPT6	***Oryza sativa***	**AB239803**
OsIPT7	***Oryza sativa***	**AB239804**
OsIPT8	***Oryza sativa***	**AB239805**

6.1.7 *CKX* genes used for bioinformatics

Gene name	Species	Accession No
OsCKX1	***Oryza sativa***	**XM_464684**
OsCKX2	***Oryza sativa***	**gi|66274568**
OsCKX3	***Oryza sativa***	**gi|21956562**
OsCKX4	***Oryza sativa***	**gi|20161316**
OsCKX5	***Oryza sativa***	**gi|21715992**
OsCKX6	***Oryza sativa***	**gi|46805838**
OsCKX7	***Oryza sativa***	**gi|46805838**
OsCKX8	***Oryza sativa***	**gi|57834113**
OsCKX9	***Oryza sativa***	**gi|42558285**
OsCKX10	***Oryza sativa***	**gi|54291232**
OsCKX11	***Oryza sativa***	**gi|42409493**
PsCKX1	***Pisum sativum***	**EF030477**
PvCKX	***Phaseolus vulgaris***	**CV532819**
AtCKX1	***Arabidopsis thaliana***	**NM_129714.2_**
AtCKX2	***Arabidopsis thaliana***	**NM_127508.2**
AtCKX3	***Arabidopsis thaliana***	**NM_125079.2**
AtCKX4	***Arabidopsis thaliana***	**NM_179139.1**
AtCKX5	***Arabidopsis thaliana***	**NM_106199.5**
AtCKX6	***Arabidopsis thaliana***	**NM_116209.3**
AtCKX7	***Arabidopsis thaliana***	**NM_180532.2**
RfCKX1	***Rhodococcus fascians***	**Z29635**

6.2 Recipes

6.2.1 25 x TAE Buffer

121g Tris base

28.5ml glacial acetic acid

9.3g EDTA

Made up to 1L with distilled H_2O

6.2.2 1 M Tris Stock

121 g Tris bace

Made up to 1 L with nanopure water, Adjust pH to 8.0 with HCL

6.2.3 0.5 M EDTA stock

146 g EDTA

Made up to 1 L with nanopure water, Adjust pH to 8.0 with HCL

6.2.4 TE Buffer

10 ml 1 M Tris (pH 8)

2 ml 0.5 M EDTA (pH 8)

Made up to 1 L with nanopure water

6.2.5 6 x agarose gel loading dye

60mM EDTA

10mM Tris-HCl (pH 7.6)

0.03% Xylene cyanol FF

0.03% Bromophenol blue

60% glycerol

6.2.6 RT master mix

4 µl 5x RT buffer (Roche)

0.8 µl RNAsecure™ Reagent (Ambion)

1 µl 20 mM dNTPs

1 µl Expand Reverse Transcriptase, (50 U/µl) (Roche)

3.2 µl DEPC water

6.2.7 PCR

For 20 µl PCR reaction, the recipe was scaled for different reaction volumes.

2.5 µl dNTPs (2mM)

2 µl 10*Taq buffer

1 to 2 µl 25mM $MgCl_2$ (adjusted to optimise reaction)

1 µl Primers*2 (10pmol/µl)

2 µl DNA template

0.2 µl Taq (5U/µl)

Water to make up to 20 µl

6.2.8 DEPC water

DEPC is added to Nanopure water (0.1% (v/v)) and vigorously shaken to mix, then incubated on a shaker for 12 hours, then autoclaved for 45min to brake down DEPC.

6.2.9 Applied Biosystems BigDye® Terminator v3.1

For 10 µl reaction volume.

0.5 µl BDT

1.75 µl 5x sequencing buffer

1 µl (3.2 pmole) primer

3 to 10 ng purified template

Water to make up to 10 µl

Sequencing Program: 96°C for 10 s, 50°C*[1] for 10 s, 60°C*[1] variable*[2]
*[1] adjusted to match primer. *2 adjusted for sequence length

6.2.10 Sephadex

25 g Sephadex

500 ml Nanopure water

Mix Sephadex and water and stand for 10 min. Suck out water and refill, repeat until water is clear, store in the fridge. To use, suck water to 1cm above the gel and mix.

6.2.11 2×SYBR green qPCR reaction buffer

15 µl 200×SYBR green (Appendix 6.2.12)

930 µl qPCR buffer

40 µl 20 mM dNTP

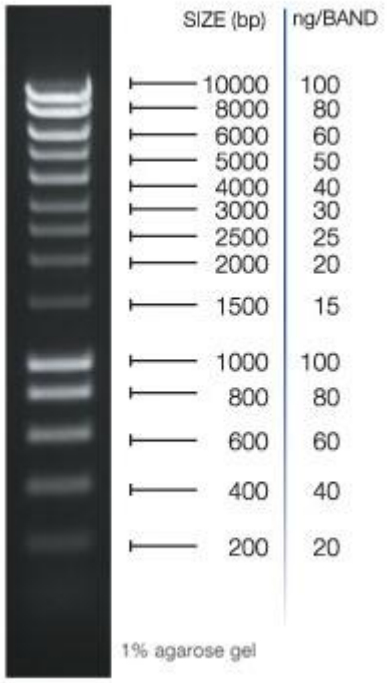
15 µl Bioline taq plomerase

6.2.12 200×SYBR green

2 µl 10,000×SYBR Green 1 (Invitrogen)

998 µl Dimethyl sulfoxide

6.3 Bioline: HyperLadder™ 1



An agarose gel run with 5 µl of HyperLadder™ 1. 2 µl of HyperLadder™ 1 was sufficient for visualisation.

6.4 Sequence Data

6.4.1 TrIPT1 sequences aligned with PCR primers

```
TrIPT1                          ---------CCCGGGAAGTCAAAGCTTTCAATTGACTTAGCAAATTATTTCCCATCAGAAATAATCAATTCAGATAAAATTCAAATTTATGAAGGTCTT [ 99]
TDipt4_primers_F1_R1            GCWACMGGRACRGGRAAGTC---------------------------------------------------------------------------- [ 99]
trIPT1_Qpcr_primers_F1_R1       ---------------------------------------------------------------------------------------------- [ 99]
TEIPT411BF_(ipt3)               ----------------------AAGCACTCG-TTGACTTGTCGA-TTATTTCC-ATC-TTGMKCCTCAATTCRSASAAAATTCYA-TCTATGAAGGTCTK [ 99]
TEIPT411CF_Psipt1/2_Atipt2/7    ----------------------AAGCTCTCG-TTGACTAG-CGA-TTATTTCCCGGA-KAGCKCCYCACTTCGGAKAAAATTCYA-TTTATGAAGGTCTT [ 99]
TEIPT411BR_(IPT3)               ---------CCCGGGAAGTCAAAGCTTTCAATTGACTTGGCAAATTATTTCCCATCAGAAATAATCAATTCAGACAAAATTCAAATCTAKGAAGGTCTT [ 99]
TEIPT411C(2)_Psipt1/2_Atipt2/7  GCWACMGGRACSGGGAAGTCAAAGCTTTCAATTGACTTAGCAAATTATTTCCCATCAGAAATAATCAATTCAGATAAAATTCAAATTTATGAAGGTCTT [ 99]

TrIPT1                          GaCATAGTAACAAACAAAATCACCAAAGAAGAACAAAAAGGAATACCyCAYCATTTACTAGGAACACATAACCCCAACAYAGAGTTCACtTCCAACGAT [198]
TDipt4_primers_F1_R1            ---------------------------------------------------------------------------------------------- [198]
trIPT1_Qpcr_primers_F1_R1       ---------ACAAACAAAATCACCAAAGAAGAAC------------------------------------------------------------- [198]
TEIPT411BF_(ipt3)               GACRTAGTARCAAACAAAATTGCMAAAGAAGAACAAAARGGAATACCRCACCATTTACTAGGAACACMTAWCCCCARCWTARAGTTCACTTCCAACGAT [198]
TEIPT411CF_Psipt1/2_Atipt2/7    GACRTAGTARCAAACAAAATCMCCAAAGAAGAACAAAARGGAATACCTCRTCATTTACTAGGAACACMTAWCCCCRRCWTAGAGTTCACTTCCAACGAT [198]
TEIPT411BR_(IPT3)               GWCATAGTAACAAACAAAATYRCMAAAGAAGAACAAAAAGGAAKACCCCMCCATTTACTAGGAACACATAACCCCAACAYAGAGTTCACWTCCAAYGAT [198]
TEIPT411C(2)_Psipt1/2_Atipt2/7  GWCATAGTAACAAACAAAATCACCAAAGAAGAACAAAAAGGAATACCYCATCATTTWCTAGGAACACATAACCCCAACAYAGAGTTCACWTCCAACGAT [198]

TrIPT1                          ttTCGCGAAAAATCAACTTYGGCCATTGATTCAATCACGAGCCGsGAACATCTTCCAATCTaCGYYGGAGGC---- [274]
TDipt4_primers_F1_R1            ------------------------------------------------GAAGGYTAGTAGCRRCCWCCR---- [274]
trIPT1_Qpcr_primers_F1_R1       ----------------------------------------GGCGCTTGTAGAAGGTTAGA---------------- [274]
TEIPT411BF_(ipt3)               TTTCGCGAAAAATCAWCTTCGGCCATTGATTCAATCACAAGCCGCGAACATCTTCCAATCT-CGYYGGAGGCATCG [274]
TEIPT411CF_Psipt1/2_Atipt2/7    TTTCGCGAAAAATCAACTTTGGCCATTGATTCAATCACGAGCCGSGAACATCTTCCAATCTA-GYYGGAGGCATCG [274]
TEIPT411BR_(IPT3)               GT-CGCGRAAAATCAAS------------------------------------------------------------ [274]
TEIPT411C(2)_Psipt1/2_Atipt2/7  GT-CGCGAAAAATCAAC------------------------------------------------------------ [274]
```

6.4.2 TrIPT3 sequences aligned with PCR primers

```
TrIPT3_Ps1_Gm1_cds_FTRC101567L23 ATGATGAATATATTTTCGGGTGCTGTTTCTTGCAAACCCCTAGTGAGTTTCCAACCGGCACTAATGGATAATAACAATTCATTGTTTCAA
TrIPT3_Primers_F1_R1             --------TATATTTTCGGGTGCTGTTTCT------------------------------------------------------------
Ipt311slg_Ipt3F1                 -------------------------------CGCAG-CCCCTAGTGAGTTTCCA-CCGGCACTWWWGGATA-TAACA-TTCATTGTTTCA-
Ipt311slg_Ipt3R1                 -------TTATATTTTCGGAGTSTGTTTCTTGCAAACCCCTAGTGAGTTTCCWACCGGCWCTAATGGATAATAACWATTCATTGTTTCAA

TrIPT3_Ps1_Gm1_cds_FTRC101567L23 CAAAAACATCGTAATCGTAATAAC---AATAAAGAGAAAGTTGTTGTTATCATGGGTGCTACCGGAACCGGGAAATCCAAGTTGGCAATT
TrIPT3_Primers_F1_R1             ---------------------------------------------------------------------------------------
Ipt311slg_Ipt3F1                 CAAAAACATCGTAATCGTAATAAC---AATAAARAGAAAGTTRTTGTTATCATGGGTGCTACCGGAACCGGGAAATCCAAGTTGGCAATW
Ipt311slg_Ipt3R1                 CAAAAACWTCGTAATCGTAATAAC--CWATAAAGAGAAAGTTRTTGTTATCWTGGGTGCTACCGGAACCGGGAAATCCRAAGCTGGCMAK

TrIPT3_Ps1_Gm1_cds_FTRC101567L23 GATATAGCAACACATTTTTCACCG-
TrIPT3_Primers_F1_R1             ctatatcgttgtgtaaaaagtggc-
Ipt311slg_Ipt3F1                 GATATAGCA-CACATTTTTCACMGA
Ipt311slg_Ipt3R1                 MTWT--------------------
```

6.4.3 TrIPT5 sequences aligned with PCR primers

```
TrIPT5_CTR0036033096-cF2_2004072 TTTCGTTCAAAATATGATTGTTGTTTCATTTGGACCGATGTGTCTTTGCCTATTCTATTTCAATATTTAGACAAAAGAGTTGATGAAATG
TrIPT5_Primers_F2_R2             ------------------------------------ATGTGTCTTTGCCTATTCTATTTC----------------------------
Ipt522_F2a                       ------------------------------------------------------------AAGTGTTTAGACAAA-GAGTTGATGAA-TG
Ipt522_R2a                       ------------------------TATTATWTTRGTTATGTGTCTTTGCYTATTCTATWTCAATATTTAGACAAAAGAGTTGATGAAATK
Ipt522_F2b                       ------------------------------------------------------------AGTATTTAGACACT-GAGTTGATGAA-TG

TrIPT5_CTR0036033096-cF2_2004072 GTTGACGCTGGGTTGGTGGATGAGATTAGAGAATTTTTTGTACCTGGAGCAAACTGTGAAGCGGGGATTAGAAGGGCAATTGGAGTTTCT
TrIPT5_Primers_F2_R2             ----------------------------------------------------------------------------------------
Ipt522_F2a                       GTTGACGCTGGGTTGGTGGATGAGATTAKAGAATTTTTTGTACCTGGAGCAAACTGTGAAGCGGGGATTAKAAGGGCAATTGGGGTTTCT
Ipt522_R2a                       GTTGACGCTGGGTTGGTGGATGAGATTAGAGAATTTTTTGTACCTGGAGCAAACTGTGAAGCGGGGATTAGAAGGGCAATTGGGGTTTST
Ipt522_F2b                       KTTGACGCTGGGTTGGTGGATGAGATTAGAGAATTTTTTGTACCTGGAGCAAACTGTGAAGCGGGGATTAGAAGGGCAATTGGGGTTTCT

TrIPT5_CTR0036033096-cF2_2004072 GAGCTCAATTATTATTTTAAGATAGAAAATGAAAAAGATATTGATGTGGATCAGAAGGAAAATATACTAAAGGAAGCAATTATAAAAACC
TrIPT5_Primers_F2_R2             ----------------------------------------------------------------------------------------
Ipt522_F2a                       GAGCTCAATTATTATTTTAAGATAGAAAATGAAAAAGATATTGATGWGGATCAGAAGGAAAATATACTAAAGGAAGCAATTATAAAAACC
Ipt522_R2a                       GAGCTCAAWTATTATTTTAAGATAGAAAATGAAAAAGATATTGATGWGGATCAGAAGGAAAATATACTAAAGGAAGCAATTATAAAAACC
Ipt522_F2b                       GAGCTCAATTATTATTTTAAGATAGAAAATGAAAAAGATATTGATGAGGATCAGAAGGAAAATATACTAAAGGAAGCAATTATAAAAACC

TrIPT5_CTR0036033096-cF2_2004072 AAACAAAACACTTGCAAATTGGCTGAAAATCAACTCTCCAAGATCCATAATATGGTTTATAATCTTGGATGGAAGATGAACAAAATTGAT
TrIPT5_Primers_F2_R2             ----------------------------------------------------------cctaccttctacttgttttaact-
Ipt522_F2a                       AAACAAAACACTTGCAAATTGGCTGAAAATCAACTCTCCAAGATCCATAATATGGTTTATAATCTTGGATGGAAGTGGAAACAAAATTGA
Ipt522_R2a                       AAACAAAACACTTGCAAAWTGGCTGAAAATCAAMWMTCCAAGATCCATAATAGKGGTTATWATTAMTT----------------------
Ipt522_F2b                       AAACAAAACACTTGCAAATTGGCTGAAAATCAACTCTCCAAGATCCATAATATGGTTTATAATCTTGGATGGAAGTGGAAACAAAATTGA

TrIPT5_CTR0036033096-cF2_2004072 TCTACAAAAGTGTTTGAGGCCATTTTAAGTGGAGAAGATTATAAACATTTGTATCAAGAGATTGTGGTTAAGCCAAGTATTGAGATTGTG
TrIPT5_Primers_F2_R2             ----------------------------------------------------------------------------------------
Ipt522_F2a                       AGTMKKRWKGAA----------------------------------------------------------------------------
Ipt522_R2a                       ----------------------------------------------------------------------------------------
Ipt522_F2b                       A---------------------------------------------------------------------------------------

TrIPT5_CTR0036033096-cF2_2004072 ACTAGATTTCTAGAGGAGACAACTCATGCAACTTGAAAT
TrIPT5_Primers_F2_R2             -----------------------------------------
Ipt522_F2a                       -----------------------------------------
Ipt522_R2a                       -----------------------------------------
Ipt522_F2b                       -----------------------------------------
```

6.4.4 TrIPT2

>TrIPT2 consensus peptide sequence
LSLKYSLSLFLPMPTTTKTTPSYLSHYPQRYYHHKPIKYRNFSHTHFSTAATRR?HFPRMEISSLHHRWKDKVIVIMGATGSGKSRLSVE?ATRFRYSEIINSDKIQVYKGLDITTNKIPFHQRNNVPHHLLGDVD?SHGE
FSPSDFRRYAGDIISDITSRRKLPIIVGGSNSFIHALLVERFDSELNVFEDESST?SSSEISSDLRYKCCFIWMDISFPVLSEYLLKRVDDMFDSGMVDELAEFYEPDADNRTGLRKAIGVPEFDRFFKQYPPV?PIEKEG
?N?SMRE?AYEEAVKAIKDNTCQLAKRQI?KILRLKRAGWDLQRIDATEAFRAVLTSESNGGGDGFSDVWKKQVLEPSMKIVNRFLME*

>TrIPT2_consensus_cds
TCACTAAAATATTCTCTCTCTCTCTTTCTTCCTATGCCTACAACAACAAAAACAACACCTTCATATCTCTCACACTACCCTCAACGTTATTATCACCACAAACCCATTAAATATCGCAACTTTTCACACACWCATTTCTCC
ACCGCCGCAACACGGCGGCMMCACTTCCCACGCATGGAAATCTCCTCCTTACACCACCGwTGGAAAGACAAAGTTATmGTCATAATGGGCGCwACCGGCTCyGGyAAATCACGTCTTTCCGTTGAAmTmGCmACyCGTTTC
CCyTACTCCGAAATAATCAACTCCGATAAAATmCAAGTyTACAAAGGACTyGATATmACCACCAACAAGATCCCCTTTCATCAACGCAACAACGTTCCTCATCATCTTCTCGGCGACGTTGACyCTTCTCACGGCGAGTTy
TCACCTTCAGATTTyCGyCGCTACGCTGGAGATATTATCTCwGATATAACTTCACGGAGAAAGCTTCCCATTATCGTTGGTGGGTCTAACTCATTCATwCACGCTCTTCTTGTAGAACGATTTGACTCAGAGTTGAACGTG
TTTGAAGACGArTCAtcaacawcaTCATCATCGGAGATATCATCGGATTTAAGGTACAAATGTTGCTTTATTTGGATGGATATATCGTTTCCTGTGTTATCGGAATATTTACTGAAACGAGTCGACGATATGTTTGACTCG
GGAATGGTGGACGAGTTAGCCGAGTTTTATGAACCGGATGCGGATAACCGAACCGGGTTAAGAAAAGCAATCGGTGTACCCGAGTTCGACCGGTTTTTTAAACAGTATCCACCGGTTrrACCCATTGAAAAAGAAGGTArT
AATratTCAATGCGGGAAskTGCATACGAGGAAGCAGTGAAGGCGATTAAAGATAACACGTGTCAGCTAGCTAAGAGACAGATAGRGAAGATCTTACGGTTAAAACGAGCTGGrTGGGACCTACAAAGAATTGATGCCACG
GAGGCGTTTAGGGCGGTGCTGACGTCAGAGTCTAACGGCGGAGGAGATGGATTTTCCGATGTATGGAAAAAGCAAGTGTTGGAACCAAGCATGAAGATTGTGAATCGTTTCTTGATGGAGTAG

6.4.5 TrIPT4

>TRIPT4 peptide sequence
MIIPVPAISSSACKQDMPLINFQKGLNKMESLFHNRNKDKVVVIMGATGTGKTKLAIDLAKHFQPAEIVNSDKMQVYKCLDITTNKVTEEECDGVPHHLLGVFDPNSNFTANDFCYHACSAIDSIVEKDGLPIIAGGSNSY
LDALVNHCSEFRLRYECCFLWVDVSFPVLHSSLSARVDRMIEAGQVNEVREFFDENDRDYTRGIRRAIGVPEFDEFFRAELEGRVDERTMKMLLEVAIDALKMNNIKLANRQVSKIRRLYGMWKRNMHRLDATDVVLKERN
WEDCVLAKSLRIVHKFLYEDSYNSRVRVGGGCGVGSSIASSSVSHQFI*

>TRIPT4_cds
AAAAGAAGAAGAAAAAAAAATGATTATTCCAGTGCCAGCAATATCATCCTCTGCCTGCAAACAAGACATGCCCTTAATAAATTTCCAAAAGGGACTCAACAAAATGGAATCATTATTTCATAATAGAAACAAAGATAAGGTG
GTAGTGATTATGGGGGCCACAGGAACTGGAAAGACCAAATTAGCCATAGATTTGGCCAAACATTTTCAACCAGCTGAAATTGTGAATTCAGATAAAATGCAAGTTTATAAATGTCTAGACATTACAACAAATAAGGTGACA
GAAGAAGAGTGTGATGGGGTCCCACACCATCTCTTAGGTGTATTTGATCCAAATTCAAATTTCACTGCCAATGATTTTTGTTATCATGCTTGTTCAGCTATTGATTCCATTGTGGAAAAAGATGGATTACCAATTATTGCT
GGTGGCTCAAATTCATATTTAGATGCTTTAGTGAATCATTGTAGTGAATTTAGGTTAAGGTACGAATGTTGTTTTCTTTGGGTTGATGTTTCATTTCCCGTGCTTCATTCGTCGTTATCCGCACGAGTTGATCGTATGATT
GAAGCTGGTCAAGTCAATGAAGTTCGTGAGTTTTTTGACGAAAATGATCGTGATTATACGAGAGGGATACGAAGAGCCATTGGTGTCCCGGAATTTGATGAATTTTTTAGAGCTGAGTTGGAAGGAAGAGTTGATGAAAGA
ACCATGAAGATGCTTCTAGAAGTTGCTATTGATGCTCTTAAGATGAATAATATTAAGCTTGCAAATAGACAAGTTTCGAAGATTCGTAGGCTTTAT-
GGTATGTGGAAAAGAAACATGCATCGTCTTGATGCTACGGATGTTGTTCTTAAGGAAAGGAATTGGGAGGATTGTGTTTTGGCTAAGAGTCTTAGGATTGTTCATAAATTTCTATACGAGGATAGTTACAATTCTCGTGTT
CGTGTTGGTGGTGGTTGTGGTGTTGGGTCATCAATCGCTTCCTCGAGTGTGAGTCATCAATTCATATAAGGAGTTGGTCAAATCATTCGTTGA

6.4.6 IPT Peptide alignment

```
TrIPT1            ----------------------------------------------------------------------------------------PGK [100]
TrIPT2            ---------LSLKYSLSLFLPMPTTTKTTPSYLSHYPQRYYHHKPIKYRN-------FSHTHFSTAATRRHHFPRMEISSLHHRWKDKVIVIMGATGSGK [100]
TrIPT3            -------MMNIF----------------SGAVS-CKPLVSFQPALM-----------------DNNNSLFQQKHRNRNNNK-----EKVVVIMGATGTGK [100]
TRIPT4            KRRRKKMIIPVPA------------IS-SSACKQDMPLINFQKGLN-----------------KMESLF-HNRNK-----------DKVVVIMGATGTGK [100]
TrIPT5            ---------------------------------------------------------------------------------------- [100]
ATIPT1            MTELNFHLLPIISDRFTTTTTTSPSFSSHSSSSSSLLSFTKRRRKHQP---------------LVSSIRMEQSRSRNRK-------DKVVVILGATGAGK [100]
AtIPT3            ------MIMKIS-------------MAMCKQPLPPSPTLDFPPARFG-----------------PNMLTLNPYGPK----------DKVVVIMGATGTGK [100]
AtIPT4            ----------MK-------------CN-------------------------------------------------------DKMVVIMGATGSGK [100]
AtIPT5            ----------MKP-------------CMTALRQVIQPLSLNFQGN-----------------MVDVPFFRRK-------------DKVVFVMGATGTGK [100]
AtIPT6            -------MQQLMT------------LLSPPLSHSSLLPTVTTKFGSPR----------------LVTTCMGHAGRKNIK--------DKVVLITGTTGTGK [100]
ATIPT7            --------MKFS----------------ISSLKQVQPILCFKNKLSK----------------VNVNSFLHPK-------------EKVIFVMGATGSGK [100]
LjIPT1            --------MRLSS------------LSPHPHHHHHYTTHYHYHYHH-----------------PSSLAMDGHRRI----------DKVVVIMGATGSGK [100]
LjIPT2            --------MNIFS-------------VFASSSYKPLVMSNFQPALTT----------------TMDSLFHHSK-------------DKVVVIMGATGSGK [100]
LjIPT3            --------MSIS----------------MLMCRLRQPLINVPCSGK-----------------KLSMRQIQK-------------EKVVLVMGATGTGK [100]
LjIPT4            ------MIIPVPP----------------SPCKQELPLVNFQNGL-----------------IMESLFRHHRNK-----------DKVVVIMGATGTGK [100]
PsIPT1            -------MMNIFS------------VV-SGAASACKPMVSFQPPLM-----------------DNDNLRFHQQHRK----------EKVVVVMGATGTGK [100]
PsIPT2            ------MIIPVAA------------TS-SSACKQELPLINFQKGLT-----------------KMESLF-HNRNK-----------DKVVVIMGATGTGK [100]
GmIPT             ------MNISTSA------------CACACACKQELPLVSFQKGSL-----------------MMESLF-HHRNNSNK--------DKVVVIMGATGAGK [100]
OsIPT1            ----------MEH------------CNGIAAVGRWLSTK-------------------------------------------------PKVIFVLGATATGK [100]
OsIPT2            ----------MEY-------------HVGGVIGQS-PK-------------------------------------------------PKVVFVLGATATGK [100]
OsIPT3            ------------------------MEGSR-GD--------K-------------------------------------------GKVVVVMGATATGK [100]
OsIPT4            -------MQAYMA------------VAAAPAPPASLTLLPRTTTVIRDRE-------------RFDAAVPVAPLVLRHGAGVK---HKAVVVMGATGTGK [100]
OsIPT5            -----MATSLSLA----------PKPAAVAVAAAAIPR--LVPPPSIDMS-------------ALSPPPPLVSVS--RSMVAK---HKAVVVMGATGTGK [100]
OsIPT7            -------MTSVATR---------IATLVRAAAAASRPLRLHRRPGGED---------------------------------------TRMVVIVGATGTGK [100]
OsIPT8            ------------------------MERSRVGDGCCCSCSGRGGVAS-----------------TTAVRPS---------------TGMVVIVGATGTGK [100]
ZmIPT1            --------MAHPS----------SAAAVSSTAPAANPSYGAREEGG-----------------ARSPPSPSPSPSQRGR-------AKVVIVMGATGAGK [100]
ZmIPT2            ------------------------MEHGAVAGK-------------------------------------------------PKVVFVLGATATGK [100]
ZmIPT4            -------MSLYLA----------PTAAAATTTTTTLPRQ-LLPAPSIDLRRLERRGMALPLPPAPAPPPPLVSAN-NRHAGAK---HKAVVVMGATGTGK [100]
ZmIPT5            ----------MAA----------PAMAAPPPPPACFPMPTRLTMPPTSIT-------------LPDPP-PLSVGGACRRVAAK---HKAVVVLGATGTGK [100]
ZmIPT6            ------MTLSMTA----------PAMAAAP--PACSPMAARLTMPP-----------------LPDAAAPLSVVG-CRRMAAK---HKAVVVLGATGTGK [100]
ZmIPT7            ----------MAG------------VNGATASGGDNK-------------------------------------------------AKVVLVMGATATGK [100]
ZmIPT8            ------MTTLLANRITTLVRAPPPPMAAAAVAGARRPLHRTLAHPPPPEEDEHQQQRACRSRGSSSSCSASSSSTPARPRG-----TGMVVIVGATGTGK [100]
R.fascians_IPT    ------------------------MKESTMAQTQARFDRVR-----------------------------------------WEPGVYAIVGATGIGK [100]
A_tumefaciens_IPT ------------------------------------------------------------------------------------MDLRLIFGPTCTGK [100]
```

```
TrIPT1              SKLSIDLAN-YFPSEIINSDKIQIYEGLDIVTNKITKEEQKGI??HLLGTHNPN-?EFTSNDFREKST?AIDSITS?EHLPIY?GG-------------- [200]
TrIPT2              SRLSVEIATRFPYSEIINSDKIQVYKGLDITTNKIPFHQRNNVPHHLLGDVDSSHGEFSPSDFRRYAGDIISDITSRRKLPIIVGGSNSFIHALLVERFD [200]
TrIPT3              SKLAIDIATHFSP---------------------------------------------------------------------------------- [200]
TRIPT4              TKLAIDLAKHFQPAEIVNSDKMQVYKCLDITTNKVTEEECDGVPHHLLGVFDPN-SNFTANDFCYHACSAIDSIVEKDGLPIIAGGSNS----------- [200]
TrIPT5              ---------------------------------------------------------------------------------------------- [200]
ATIPT1              SRLSVDLATR-FPSEIINSDKIQVYEGLEITTNQITLQDRRGVPHHLLGVINPEHGELTAGEFRSAASNVVKEITSRQKVPIIAGGSNSFVHALLAQRFD [200]
AtIPT3              SRLSVDIATRFRA-EIINSDKIQVHQGLDIVTNKITSEESCGVPHHLLGVLP-PEADLTAANYCHMANLSIESVLNRGKLPIIVGGSNSYVEALVDDKEN [200]
AtIPT4              SSLSVDLALH-FKAEIINSDKMQFYDGLKITTNQSTIEDRRGVPHHLLGELNPEAGEVTAAEFRVMAAEAISEITQRKKLPILAGGSNSYIHALLAKSYD [200]
AtIPT5              SRLAIDLATRFP-AEIVNSDKIQVYKGLDIVTNKVTPEESLGVPHHLLGTVHDTYEDFTAEDFQREAIRAVESIVQRDRVPIIAGGSNSYIEALVNDCV- [200]
AtIPT6              SRLSVDLATRFFPAEIINSDKMQIYKGFEIVTNLIPLHEQGGVPHHLLGQFHPQDGELTPAEFRSLATLSISKLISSKKLPIVVGGSNSFNHALLAERFD [200]
ATIPT7              SRLAIDLATRFQ-GEIINSDKIQLYKGLDVLTNKVTPKECRGVPHHLLGVFDSEAGNLTATQYSRLASQAISKLSANNKLPIVAGGSNSYIEALVNHSS- [200]
LjIPT1              SRLSIDLATLFPFSEIINSDKMQVYKGLDTTTNKIPPHQRNNVPHHLLGDVDPSLGDFTPSDFRRRAGDLISDITRRRKLPIFVGGSNSFVHALLVDRFD [200]
LjIPT2              SKLAIDLATHFPPAEVVNSDKIQMYSGLDIATNKVTEEECRGVPHHLLGTFENPNMNFTASDFCKQASSAIGSIVKRDGLPIIAGGSNSFIDALVNHTP- [200]
LjIPT3              SKLSIDLATCFPS-EIINSDKIQIYDGLDIVTNKISKEEQRGIPHHLLGTQN-PNTDFTAGDFSDCSTAAIDAITSRDHLPIIAGGSNSYLEALIDDDDY [200]
LjIPT4              TKLAIDLAKHFQPAEIVNSDKIQVYKGLDITTNKVTEEECGGIPHHLLGAVDDPNYNFTANDFCHHACSAIDSIVKKDGLPIIAGGSNSYLDALVNHHA- [200]
PsIPT1              SKLAIDLANHFPPAEIINSDKMQVYKGLDITTNKVTEEECRGVPHHLLGTAD-PDSSFTSNEFCEHATLAIGSIVGRDGLPIIAGGSNSFIEALVNHHH- [200]
PsIPT2              TKLAIDLAKHFQPSEIVNSDKIQVYKGLDITTNKVTEQECDGVPHHLLGSFD-PTTNFTANDFCYHACSAIDSIVQKDGLPIIAGGSNSYLDTLVNHCS- [200]
GmIPT               TKLAIDVAKHFQPAEIVNSDKMQVYKGLDITTNKVTEEECGGVPHHLLGTVD-PYINFSANDFCRYATLAIDSIVEKNGLPIIAGGSNSYLDALVNHYP- [200]
OsIPT1              SKLAIRLAARFD-GEVINSDKIQAHDGFPVITNKVTDEERAGVAHHLLGGVSPDA-DFTAEDFRREAAVAVARVHAAGRLPVVAGGSNIYVEALVAGGGG [200]
OsIPT2              SKLAISIAERFG-GEVINSDKIQVHDGFPIITNKVTEEERAGVPHHLLGVLHPDA-DFTAEDFRREAAAAVARVLAAGRLPVVAGGSNTYVEALVEGGGG [200]
OsIPT3              SKLAIDLALRFG-GEVINSDKIQVHDGLDVVTNKVTDEERAGVPHHLIGGVPPDA-DYGVDDFRRDAARAVASVLARGRVPVIAGGSNRYLEALLDGEGG [200]
OsIPT4              SRLAVDLALRFG-GEVINSDKMQIHSGLDVVTNKVTEEECAGVPHHLISVARPDD-EFTAADFRREAARAAAGAVERGRLPIIAGGSNSYVEELVEGDGR [200]
OsIPT5              TRLAVDLALQFG-GEVINADKLQLHRGLDVATNKATADERAGVPHHLIGVAHPDE-EFTAADFRRAASRAAAAVAARGALPIIAGGSNSYIEELVDGDRR [200]
OsIPT7              TKLSIDAAKVIG-GEVVNADKIQLYDGLDVTTNKVSLADRRGVPHHLLGAIRPEAGELPPSSFRSLAAATAASIAARRLVPVIAGGSNSLIHALLADHFD [200]
OsIPT8              TKLSIDAAQELA-GEVVNADKIQLYDGLDVTTNKVSLADRRGVPHHLLGAIRAEAGELPPSSFRSLAAAAAAGIASRGRVPVVAGGSNSLIHALLADPID [200]
ZmIPT1              SRLAVDLAAHFAGVEVVSADSMQLYRGLDVLTNKAPLHEQNGVPHHLLSVIDPSV-EFTCRDFRDRALPIIQEIVDRGGLPVVVGGTNFYIQALVSPFLL [200]
ZmIPT2              SKLAIALAERFN-GEVINADKIQVHDGVPIITNKVTEEEQGGVPHHLLSVRHPDA-DFTAEEFRREAASAVARVLSAGRLPVVAGGSNTYIEALVE---- [200]
ZmIPT4              SRLAVDLALRFG-GEVINSDKIQLHAGLDVTTNKVTEQERAGVPHHLLGVARPDE-EFTAADFRREATRAARAITARGRLPIVAGGSNSYVEELVD---- [200]
ZmIPT5              SRLAIDLALRFG-GEVINSDKIQAHAGLDVATNKVGLAERGRVPHHLLGVVHPDA-EFTAADFRREASRAADRAAARGRVPVIAGGSNSYVEELVE---- [200]
ZmIPT6              SRLAIDLALRFG-GEVINSDKIQAYAGLDVATNKVGPAERAAVPHHLLGVVHPDA-EFTAADFRREAAGAAARVASRGRVPIIAGGSNSYVEELVE---- [200]
ZmIPT7              SKLAIDLALRFG-GEVVNSDKIQVHDGLDVVTNKVTAAERQGVPHHLIDGVAPDA-DYTTADFCRDAVRAVESILERGRVPIIAGGSNRYLEALLD---- [200]
ZmIPT8              TKLSIDAAEAVG-GEVVNADKIQLYAGLDVTTNKVAPADRRGVPHHLLGAIRPEAGELPPSTFRSLAAATAASIAARGRLPVVAGGSNSLIHALLADRLD [200]
R.fascians_IPT      SAEASKLALSHS-APIVVADRIQCYSDLLVTSGRAFDAKVEGLNRVWLDNRTIHQGNFDPDEAFDRLIKVLTSYVDRGEAVVMEGGSISLILRFAQTISN [200]
A_tumefaciens_IPT   TSTAVALAQQTG-LPVLSLDRVQCCPQLSTGSGRPTVEELKGTSRLYLDDRPLVKGIIAAKQAHERLMGEVYNYEAHGGL-ILEGGSISLLKCMAQSSYW [200]
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TrIPT1            ---------------------------------------------------------------------------------------------------- [300]
TrIPT2            SELNVFEDE------------------------------------------------------------SSTSSSSEISSDLRYKCCFIWMDISFPVLS [300]
TrIPT3            ---------------------------------------------------------------------------------------------------- [300]
TRIPT4            ------------------------------------------------------------------YLDALVNHCSEFRLRYECCFLWVDVSFPVLH [300]
TrIPT5            ---------------------------------------------------------------------------------FRSKYDCCFIWTDVSLPILF [300]
ATIPT1            PKFDPFSSG--------------------------------------------------------------------SCLISSDLRYECCFIWVDVSETVLY [300]
AtIPT3            ----KFRSR----------------------------------------------------------------------YDCCFLWVDVALPVLH [300]
AtIPT4            PENYPFSDH--------------------------------------------------------------------KGSICSELKYDCCFIWIDVDQSVLF [300]
AtIPT5            ----DFRLR----------------------------------------------------------------------YNCCFLWVDVSRPVLH [300]
AtIPT6            PDIDPFSPG--------------------------------------------------------------------SSLSTICSDLRYKCCILWVDVLEPVLF [300]
ATIPT7            ----GFLLN-----------------------------------------------------------------N----------YDCCFIWVDVSLPVLN [300]
LjIPT1            PESNVFRDD--------------------------------------------------------------------S-PSPVSSELRYRCCFLWMDIAFPVLS [300]
LjIPT2            ----EFRFN----------------------------------------------------------------------YECCFLWVDVSLPVLH [300]
LjIPT3            ----KFRSR----------------------------------------------------------------------YDFCCLWVDVAMPVLD [300]
LjIPT4            ----EFRLR----------------------------------------------------------------------YECCFLWVDVSLPVLH [300]
PsIPT1            ----EFRMK----------------------------------------------------------------------YECCFLWVDVSIPVLH [300]
PsIPT2            ----EFRLR----------------------------------------------------------------------YECCFLWVDVALPVLH [300]
GmIPT             ----EFRLR----------------------------------------------------------------------YQCCFLWVDVALPVLH [300]
OsIPT1            AFL----------------------------------------------------------------------------AAYDCLFLWTDVAPDLLR [300]
OsIPT2            AFR----------------------------------------------------------------------------AAHDCLFLWTDVAPGLLR [300]
OsIPT3            SFR----------------------------------------------------------------------------ERHELCFLWVDSRAPALH [300]
OsIPT4            AFR----------------------------------------------------------------------------ERYECCFLWVDVDLEVLR [300]
OsIPT5            AFR----------------------------------------------------------------------------DRYDCCFLWVDVQLPVLH [300]
OsIPT7            ASAGDPFSPAAAF-----------------------------------------------------------RHYRPALRFPCCLLWVHVDEALLD [300]
OsIPT8            AAPRDPFADADVG-----------------------------------------------------------YRPALRFPCCLLWVDVDDDVLD [300]
ZmIPT1            DDMAEEMQGCTLRDHIDDGLTDEDEGNGFERLKEIDPVAAQRIHPNDHRKIKRYLELYATTGALPSDLFQGEAAKKWGRPSNSRLDCCFLWVDADLQVLD [300]
ZmIPT2            ------------------------------------------------------------GDG-----AAFRAAHDLLFVWVDAEQELLE [300]
ZmIPT4            ------------------------------------------------------------GDR-----AAFRDRYDCCFLWVDVQRAVLH [300]
ZmIPT5            ------------------------------------------------------------GDR-----RAFRDRYECCFLWVDAQLPVLH [300]
ZmIPT6            ------------------------------------------------------------GDR-----RAFRERYDCCFLWVDARLPVLH [300]
ZmIPT7            ------------------------------------------------------------GEPP----AGFRGRYECCFLWVDSDLAV?D [300]
ZmIPT8            AG-----------------------------------------------------AADPFSAPPQPAPPRWGRRPALRSPCCLLWVHVDAALLA [300]
R.fascians_IPT    ---------------------------------------------------------------------------------------LPFPAVV [300]
A_tumefaciens_IPT S------------------------------------------------------------------------ADFRWDIIRHELADEETFMNVAK [300]
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TrIPT1              ------------------------------------------------------------------------------------------ [400]
TrIPT2              EYLLKRVDDMFDSGMVDELAEFYEPD-----ADNRTGLRKAIGVPEFDRFFKQYPPV------KPIEKEGSN-SMRERAYEEAVKAIKDNTCQLAKRQIE [400]
TrIPT3              ------------------------------------------------------------------------------------------ [400]
TRIPT4              SSLSARVDRMIEAGQVNEVREFFDEN----DRDYTRGIRRAIGVPEFDEFFR------------AELEGRVDERTMKMLLEVAIDALKMNNIKLANRQVS [400]
TrIPT5              QYLDKRVDEMVDAGLVDEIREFFVPG-----ANCEAGIRRAIGVSELNYYFK------------IENEKDIDVDQKENILKEAIIKTKQNTCKLAENQLS [400]
ATIPT1              EYLLRRVDEMMDSGMFEELSRFYDPVKS-GLETR-FGIRKAIGVPEFDGYFKEYPPEKK--------MIKWDALRK-AAYDKAVDDIKRNTWTLAKRQVK [400]
AtIPT3              GFVSERVDKMVESGMVEEVREFFDFS----NSDYSRGIKKAIGFPEFDRFFRNEQF--------------LNVEDREELLSKVLEEIKRNTFELACRQRE [400]
AtIPT4              EYLSLRLDLMMKSGMFEEIAEFHRSKK---APKEPLGIWKAIGVQEFDDYLKMYKWDND--------MDKWDPMRK-EAYEKAVRAIKENTFQLTKDQIT [400]
AtIPT5              SFVSERVDKMVEMGLVDEVRRIFDPSS----SDYSAGIRRAIGVPELDEFLR-SEMRN------------YPAETTERLLETAIEKIKENTCLLACRQLQ [400]
AtIPT6              QHLCNRVDQMIESGLVEQLAELYDPVVD---SGRRLGVRKTIGVEEFDRYFRVYPKEMD--------KGIWDLARK-AAYEETVKGMKERTCRLVKKQKE [400]
ATIPT7              SFVSKRVDRMMEAGLLEEVREVFNPKA----N-YSVGIRRAIGVPELHEYLR-NES-L------------VDRATKSKMLDVAVKNIKKNTEILACRQLK [400]
LjIPT1              EYLLKRVDDMLDSGMVDELAQFFDSDT-----ANQTGLRKAIGVPEFDRFFK--------------------DPVREGAAYEEAVRAIKENTCQLAKRQIG [400]
LjIPT2              NSLSRRVDRMIDAGQVDEVRQFFLNAHH--QSDYTRGIRRAIGVPEFDRFLRAEASG-------------ADERTKRKLLDTAIAELKVNNCNLASRQVQ [400]
LjIPT3              SYVAARVDQMLRSGMVEELRPFFNA-----NGDYSRGIRRAIGVPEFDEYFRREGF--------------ADEETRKLLLERAVREMKVNTCKLARRQLG [400]
LjIPT4              SSLQARADRMIEAGQVDEVREFFDPS-----ADYTRGIKRAIGVPEFDDFLRAEANG-------------EDDMTILRLLEGAIARTKINNCTLANRQVQ [400]
PsIPT1              SSLSARVDRMIEAGQVNEVRDFFNQKNF--DYDYTRGIRRAIGVPEFDKFFRNESQGV------------TDERTMKKLLEVAVDALKMNNCNLASKQVQ [400]
PsIPT2              SSLQSRVDRMIEAGQVDEVREFFDPS-----GDYTKGIRRAIGVPEFHDFLTAEANS-------------ADERTKKKLLEAAITRVKINNCTLANRQRQ [400]
GmIPT               SSLQARVDRMIEAGQVNDVRDFFDPSVT----DYTKGIRRAIGVPEFDDFLRAEANG------------RLDERTKQRLLQAAIARLKINNCTLANRQIQ [400]
OsIPT1              WYTAARVDDMVRRGLVGEARAGFDAGA-----DYTRGVRRAIGLPEMHGYLLAEREGG--------AGAEDDDDLLAGMLEAAVREIKDNTFRLTVSQVA [400]
OsIPT2              WYTAARVDDMVRRGLVGEARAGFVDGAG-AADYYTRGVRRAIGIPEMHGYLLAERSGGE--------AADDGE--LAAMLDGAVREIKANTYRLAATQVA [400]
OsIPT3              RYVRHRVDRMVEQGLVGEVRGLFRLDD----ADYSRGIRRSIGVPEMDAYLRQEATG---------ALLTHGDKYKVALLASAVGEIKANTWSLARRQLR [400]
OsIPT4              GFVARRVDEMCRRGLVREVAAAFDPRR----TDYSRGIWRAIGVPELDAYLRSRGDG-------------ADEEERARMLAAAVAEIKLNTFRLACRQHR [400]
OsIPT5              GFVGRRVDDMCGRGMVAEIEAAFDPDR----TDYSRGVWRAIGVPELDAYLRSCAAA-------------GGEEERARLLANAIEDIKANTRWLSCRQRA [400]
OsIPT7              EYLDRRVDDMVDAGMVEELREYFATTTA-AERAAHSGLGKAIGVPELGDYFAG-----------------------RKTFSEAIDDIKANTRVLAAAQVS [400]
OsIPT8              EYLDRRVDDMVGEGMVEELEEYFATTSA-SERASHAGLGKAIGVPELGDYFAG-----------------------RKSLDAAIDEIKANTRVLAARQVG [400]
ZmIPT1              SYVNKRVDCMMDGGLLDEVCSIYD-----ADAVYTQGLRQAIGVREFDEFFRAYLPRKESGEGSCASLLGMHDDQLKSLLDEAVSQLKANTRRLVRRQRR [400]
ZmIPT2              WYAALRVDEMVARGLVSEARAAFGG----AGVDYNHGVRRAIGLPEMHAYLVAEREGVAG------------EAELAAMLERAVREIKDNTFRLARTQAE [400]
ZmIPT4              GCVARRVDEMRARGLVDEVAAAFDP----RRNDYSRGLWRAIGAPELDAYLRWPGPGVDGDAE--------SEGERDRLLAAAIEDIKSNTRRLSCRQRA [400]
ZmIPT5              GFVARRVDDMCRRGLVDEVAAAFDP----RRTDYSRGIWRAIGVPELDAYLRARGR-GHG-------------HHHDQMLAAALHEIKANTSRLAVRQRG [400]
ZmIPT6              GFVARRVDEMCRRGLVDEVAAAFDP----RRTDYSRGIWRAIGVPEMDAYLRAGGH-GDGDGD--------EQEQRARMLAAALDEIKVNTSRLALRQRG [400]
ZmIPT7              RYIGSRVDCMLEQGLVREVRAFFRH----DDADYSRGIRRAIGVPEMDMYFRMEAAGALDGDD--------DDQLRVRLLAAAVNEIKANTCGLARRQLQ [400]
ZmIPT8              EYLDRRVDDMVRGGMVEELREYFAATTAAERAAHAAGLGRAIGVPELGACFAGRAS-----------------------FRAAIDDIKANTRDLAAAQVR [400]
R.fascians_IPT      NVMPIPDRQHYFAQQCARARQMLRGDS----------TGRNLLTELAEAWVLGDQHN---------------FIASVAGLDCVLDWCATHSVTPEELANR [400]
A_tumefaciens_IPT   ARVKQMLRPAAGLSIIQELVDLWKEPR-------LRPILKEIDGYRYAMLFASQNQ---------------ITSDMLLQLDADMEDKLIHGIAQEYLIHA [400]
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TrIPT1            ---------------------------------------------------------------------------------------------- [500]
TrIPT2            KILRLKR-AG-WDLQRIDATEAFRAVLTSESN--GGGDGFSDVWKKQVLEPSMKIVNRFLME*------------------------------------ [500]
TrIPT3            ---------------------------------------------------------------------------------------------- [500]
TRIPT4            KIRRLYGMWK-RNMHRLDATDVVL---------------KERNWEDCVLAKSLRIVHKFLYEDSYNSRVRVGGGCGVGSSIASSSVSHQFI*GVGQIIR* [500]
TrIPT5            KIHNMVYNLGW-KMNKIDSTKVFEAILS--------GEDYKHLYQEIVVKPSIEIVTRFLEETTHAT*N------------------------------- [500]
ATIPT1            KIEMLKDAG-W-EIERVDATASFKAVMMKSSS----EKKWRENWEEQVLEPSVKIVKRHLVQN*------------------------------------ [500]
AtIPT3            KIERLRKVKKW-SIQRVDATPVFTKRRSK--M------DANVAWERLVAGPSTDTVSRFLLDIAS-RRPLVEASTAVAAAMERELSRCLVA*-------- [500]
AtIPT4            KINKLRNAG-W-DIKKVDATASFREAIRAAKEGEGVAEMQRKIWNKEVLEPCVKIVRSHLDQPINYYYYYFYLLKRFLSLN*------------------ [500]
AtIPT5            KIQRLYKQWKW-NMHRVDATEVFLRR----GE------EADEAWDNSVAHPSALAVEKFLSYSDDHHLEGANILLPEISAVPPLP--AAVAAISR*---- [500]
AtIPT6            KIMKLIRGG-W-EIKRLDATAAIMAELNQSTAK-GEGKNGREIWEKHIVDESVEIVKKFLLEV*------------------------------------ [500]
ATIPT7            KIQRLHKKWKM-SMHRVDATEVFLKRN---VE------EQDEAWENLVARPSERIVDKFYNNNNQLKNDDVEHCLAASYGGGSGS--RAHNMI*------ [500]
LjIPT1            KIMRLKRAG-W-DLRRIDATEAFRVALVADGG----GERFSDEWKRQVLEPSVKIVKRFLME*------------------------------------ [500]
LjIPT2            KIHRLYNMWKR-SMHRLDPTEVLLKNGCCSPE------EAEKVWEDHVFSKSRRIIQKFLYEETTHRVASKNNGVISSSSSNSSTPSAMAAVTH*----- [500]
LjIPT3            KIQRLRNVKRW-EIHRVDATPVFWKRGE----------EADEAWRKVVAEPSAMIVAQFLYKAKSDVNVVSGGFRVPAGSTESVMAAATC*--------- [500]
LjIPT4            KIHRLHRVWRR-SMHQLDATDVFLRRSSGDSA------DSEEAWQDHVLAKSLMILHNFLYKEIS--CVPTRSPQVALPAFAAVAVTH*----------- [500]
PsIPT1            KIHRLYGMWKR-NMHRLDATEVVLK---------------EDNWEDRVLAKSLRIVHKFLYEDCSHVTSG---GVVP------AKIASVAGVTH*----- [500]
PsIPT2            KIQRLNGMWKR-SMYRLDATETIIRSG---TR------ARKETWEDQVLSKSLIILYNFLYGETR-VCSRNVSPKNIIDALSGSQPTLTLSAVAAATH*- [500]
GmIPT             KIHRLHAFWKR-NMHRLDATEVFR--------------GSRDAWRDHVLAKTLIILHKFLYGEKKTPHVVPAGIVSAKDVIAAAAVLSSPPVAMAATR*- [500]
OsIPT1            KIRRLSALPGW-DVRRVDATAVVARMAEG--------APHGETWREVVWEPCEEMVSRFLETPAAAAA-------VVANGKVDVNVGDAAAGVPEAAAAA [500]
OsIPT2            KIRRLSALDGW-DVRRVDATVVVARMAEG--------APHRETWEAVVWKPCEEMVGRFLEASAAVD------------DDDNAAAGSPAALAPMTAACR [500]
OsIPT3            KIHRLRGLPGW-SLRRLDVTRVLELKVEA-----RSEAECAAAWEADVIAPAAREVGMFLHGGGNVVESGREEQPVVVEKMEVAAVGGAGAAAAAEKWCG [500]
OsIPT4            KIERLDRM--W-RARRVDATEVFRRRGHA----------ADDAWQRLVAAPCIDAVRSFLFEDQERSS-------IAAGKPPLFAAGKATSGNISVFASA [500]
OsIPT5            KIVRLDRL--W-RIRRVDATEAFRRRGGA----------ANEAWERHVAAPSIDTVRSFLHGEFTTAA-------ETTAAP------VPPPPFLPMFALA [500]
OsIPT7            KIRRMSDAWGW-PIHRLDASDTVRARLTR-----AGSAAESASWERDVRGPGLATIRSFLADQSPPPRSEGTNDYLYAMETEPEPPPPPTLPPRLLRLPR [500]
OsIPT8            KIRRMADVWGW-PIRRLDATATIRARLSG-----AGRAAEAAAWERDVRGPGLAAMRQFVG------RAD-----FNAAAVD-------QLAARSRRQ-- [500]
ZmIPT1            RLHRLSKDFGW-NLHRVDATEAFFCATDDS-------------WQKKVVKPCVDVVRRFLSDNST---------VLPSTSASDPSSRELWTQYVCEACGN [500]
ZmIPT2            KIRRLSTLDGW-DVRRIDVTPVFARKADGT-------ECHELTWKKQVWEPCEEMVRAFLEPS-----------LTAVPGVAVTEEGNAGVVATAAPAGD [500]
ZmIPT4            KIQRLA--KMW-GVRRVDATEVFRRRGDEA----------DEAWQRLVAAPCIDAVRSFLRTDDAA--------ATVASDLAVDGVVPVFAPAPAAAVAG [500]
ZmIPT5            KIQRLE--RMW-RVRRVDATEVFLKRGLAA----------DEAWQRLVAAPCIDAVRSFLLEDQQ--------------EYSSMGTAGAMLPAAVAAAAV [500]
ZmIPT6            KIQRLA--RMW-RVRRVDATEVFLKRGHAA----------DEAWQRLVAAPCIDAVRSFLLEEQ--------------EYSSSMVTASMF-ASTAAAAV [500]
ZmIPT7            KIHRLHGLQGWSDIHRLDVTEVLQLKVGNAG----NPKAQRDAWETDVVSPAARIVGMFLAVEGARDKDKDRFLLTTPKEVAVPGICTATADWFGQQLDM [500]
ZmIPT8            KIRRMADAWGW-PIQRLDASATVRARLRGAG-----PDAESACWERDVRAPGLAAIRSFLLELDG--------------GSVVDGAVVEEVEPRVRCCDV [500]
R.fascians_IPT    DLTT--EVL-----DELAASMGGRYVEHGVLQ--------QEIFLRTFGAPGVTAR*------------------------------------------ [500]
A_tumefaciens_IPT RRQE--QKF-----PRV--NAAAYDGFEGHPFGMY*---------------------------------------------------------------- [500]
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TrIPT1              ------------------------------------------ [543]
TrIPT2              ------------------------------------------ [543]
TrIPT3              ------------------------------------------ [543]
TRIPT4              ------------------------------------------ [543]
TrIPT5              ------------------------------------------ [543]
ATIPT1              ------------------------------------------ [543]
AtIPT3              ------------------------------------------ [543]
AtIPT4              ------------------------------------------ [543]
AtIPT5              ------------------------------------------ [543]
AtIPT6              ------------------------------------------ [543]
ATIPT7              ------------------------------------------ [543]
LjIPT1              ------------------------------------------ [543]
LjIPT2              ------------------------------------------ [543]
LjIPT3              ------------------------------------------ [543]
LjIPT4              ------------------------------------------ [543]
PsIPT1              ------------------------------------------ [543]
PsIPT2              ------------------------------------------ [543]
GmIPT               ------------------------------------------ [543]
OsIPT1              VVAAGVV*---------------------------------- [543]
OsIPT2              LRAQLVQLQY*------------------------------- [543]
OsIPT3              RRLLETTAAYHGMEAAAAV*---------------------- [543]
OsIPT4              AAAMAAAAAI*------------------------------- [543]
OsIPT5              AAGAGV*----------------------------------- [543]
OsIPT7              MQYCDMVG*--------------------------------- [543]
OsIPT8              CLRGGMVAG*-------------------------------- [543]
ZmIPT1              RVLRGAHEWEQHRQGRGHRKRVQRLKQKSLRPWPSLLPQDRS* [543]
ZmIPT2              VVVPTGDVVTAVADA*-------------------------- [543]
ZmIPT4              *----------------------------------------- [543]
ZmIPT5              *----------------------------------------- [543]
ZmIPT6              *----------------------------------------- [543]
ZmIPT7              TVMSPSKGFAGLGSAAAAV*---------------------- [543]
ZmIPT8              VG*--------------------------------------- [543]
R.fascians_IPT      ------------------------------------------ [543]
A_tumefaciens_IPT   ------------------------------------------ [543]
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6.4.7 CKX1

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T._repens_EF691439        AATAAACTGAAAAAACGAAACCCTTTCGCTTCACATTTCACACCACAAACAAACATGATAGCCTACCTTGAACACTTCGTTCACGGAAAC
TrCKX1_concencus_sequence ----------------------------------------------------------------------------TTCACGGAAAC
TD_CKX2_F1_R1             ----------------------------------------------------------------------------------------
TDCKX211R                 ----------------------------------------------------------------------------------------
TDCKX211F                 ----------------------------------------------------------------------------------------
TrCKX1_primers_F1_R1      ----------------------------------------------------------------------------TCACGGAAAC
TrCKX111f(5)              ----------------------------------------------------------------------------------------
TrCKX111r(6)              ----------------------------------------------------------------------------------------
TrCKX111F(1)              ----------------------------------------------------------------------------------------
TrCKX111R(2)              ----------------------------------------------------------------------------TTCACGGAAAC
TrCKX111F(3)              ----------------------------------------------------------------------------------------
TrCKX111R(4)              ----------------------------------------------------------------------------TTCASGGAAAC

T._repens_EF691439        GACACAGAATCAACTCCAAACGACGACGTTTCTTCCCTCCAAGGTTCTTTCCATTTCTCCCCAATTTCCATCGCAACCAAAGACTTCGGC
TrCKX1_concencus_sequence GACACAGAATCAACTCCAAACGACGACGTTTCTTCCYTCCAAGGTTCTTTCCATTTCTCCCCAATTTCCATCGCAACCAAAGACTTCGGC
TD_CKX2_F1_R1             ------------------------CATGGHCAYTCMCTYCAAGG-----------------------------------------
TDCKX211R                 ----------------------------------------CAAGGTTCTTTCCATTTCTCCCCAATTTCCATCGCAACCAAAGACTTCGGC
TDCKX211F                 ----------------------------------------------------------------------------AGACTTCGGC
TrCKX1_primers_F1_R1      GACACAGAATC-------------------------------------------------------------------------------
TrCKX111f(5)              --------------------CGACGACGTTTCTTCCCTCCA-GGTTCTTTCCATTTCTCCCCAATTTCCATCGCAACCAAAGACTTCGGC
TrCKX111r(6)              ----------------------------------------------------------------------------------------
TrCKX111F(1)              -----------GACTCCA-CCGACGACGTTTCTTCCCTCCC-GGTTCTTTCM-TTTCTCCCCA-TTTCCRTCGCAMCCRA-GACTTCGGC
TrCKX111R(2)              GACACAGAATCAACTCCAAACGACGACGTTTCTTCCYTCCAAGGTTCTTTCCATTTCTCCCCAATTTCCATCGCAACCAAAGACTTSGGC
TrCKX111F(3)              -----------GACTCCAATCGACGACGTTTCTTCCCTCCAAGGTTCTTTCCATTTCTCCCCAATTTCCATCGCAACCAAAGACTTCGGC
TrCKX111R(4)              GACACAGAATCAACTCCAAACGACGACGTTTCTTCCYTCCAAGGTTCTTTCCATTTCTCCCCAATTTCCATCGCAACCAAAGACTTSGGC

T._repens_EF691439        GGCATGAAATCCTCCACTCCCCTCGCCTTAATCCGCCCTTACTCCACCTCCGACGTCGCAAGAGCAGTCAAAGCAGCAACAACAACCACA
TrCKX1_concencus_sequence GGCATGAAATCCTCCACTCCCCTCGCCKTAATCCGCCCTTACTCMACCTCCGACGTCGCAAGAGCAGTCAAAGCAGCAACAACAACCACA
TD_CKX2_F1_R1             ----------------------------------------------------------------------------------------
TDCKX211R                 GGCATGAAATCCTCCACTCCCCTMGCCGTAATCCGCCCTTACTCMACCTCCGACGTCGCAAGAGCAGTCAAAGCAGCAACAACAACCACA
TDCKX211F                 GGCATGAAATCCTCCACTCCCCTCGCCKTAATCCGCCCTTACTCMACCTCCGACGTCGCAAGAGCAGTCAAAGCAGCAACAACAACCACA
TrCKX1_primers_F1_R1      ----------------------------------------------------------------------------------------
TrCKX111f(5)              GGCATGAAATCCTCCACTCCCCTCGCCKWAATCCGCCCTTACTCMMCCTCCGACGTCGCAAGAGCMGTCAAAGCAGCAACAACAACCACA
TrCKX111r(6)              ------------------------------------------------------------GCAAGAGGGGTCAAAGGAGCAACAACAACCACA
TrCKX111F(1)              GGCRTGAAATCCTCCACTCCCCTCGCCKTAATCCGCCCTTACTCCACCTCCGACGTCGCAAGAGCAGTCAAAGCAGCAACAACARCCACR
TrCKX111R(2)              GGCATGAAATCCTCCACTCCCCTCGCCKTAATCCGCCCTTACTCMACCTCCGACGTCGCAAGAGCAGTCAAAGCAGCAACAACAACCACA
TrCKX111F(3)              GGCATGAAATCCTCCACTCCCCTCGCCKTAATCCGCCCTTACTCMACCTCCGACGTCGCAAGAGCMGTCAAAGCAGCAACAACAACCACA
TrCKX111R(4)              GGCATGAAATCCTCCACTCCCCTCGCCKTAATCCGCCCTTACTCMACCTCCGACGTCGCAAGAGCAGTCAAAGCAGCAACAACAACCACA
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T._repens_EF691439        AACCTAACCGTCGCGCCACGTGGCAACGGTCATTCAATCAACGGCCAAGCCATGGCGGAAGAAGGATTAGTCCTYGACATGCGCGCCACC
TrCKX1_concencus_sequence AACCTAACCGTCGCGCCACGTGGCAACGGTCATTCAATCAACGGCCAAGCCATGGCGGAAAAAGGATTAGTCCTCGACATGCGCGCCACC
TD_CKX2_F1_R1              ----------------------------------------------------------------------------------------
TDCKX211R                 AACCTAACCGTCGCGCCACGTGGCAACGGTCATTCAATCAACGGCCAAGCCATGGCGGAAAAAGGATTAGTCCTCGACATGCGCGCCACC
TDCKX211F                 AACCTAACCGTCGCGCCMCGTGGCAACGGTCATTCAATCAACGGCCAAGCCATGGCGGAAAAAGGATTAGTCCTCGACATGCGCGCCMCC
TrCKX1_primers_F1_R1      ----------------------------------------------------------------------------------------
TrCKX111f(5)              AACCTAACCGKCGCGCCACGTGGCAACGGTCATTCAATCAACGGCCAAGCCATGGCGGAAAAAGGATTAGTCCTCGACATGCGCGCCACC
TrCKX111r(6)              AACCTAACCGTCGCGCCACGTGGCAASGGTCATTCAATCAACGGCCAAGCCATGGCGGAAAAAGGATTAGTCCTCKACATGCGCGCSACC
TrCKX111F(1)              AACCTAACCGTCGCGCCMCGTGGCAACGGTCATTCAATCWACGGYCAAGCCATGGGGGAAAAAGGATTAGGCCTCGACATGSGCGCCRCC
TrCKX111R(2)              AACCTAACCGTCGCGCCACGTGGCAACGGTCATTCAATCAACGGCCAAGCCATGGCGGAAAAAGGATTAGTCCTCGACATGCGCGCCACC
TrCKX111F(3)              AACCTAACCGTCGCGCCMCGTGGCAACGGTCATTCMATCAACGGCCAAGCCATGGSGGAAAAAGGATTAGTCCTCGACATGCGCGCCACC
TrCKX111R(4)              AACCTAACCGTCGCGCCACGKGGCAACGGTCATTCAATCAACGGCCAAGCCATGGCGGAAAAAGGATTAGTCCTCGACATGCGCGCCACC

T._repens_EF691439        GCGGATGAACATTTTCAACTTCTTTATCACGAAGGTGTTCCATACGTCGACGTTTCCGGAGGGGCATTATGGGAAGAAGTGTTGAAACGC
TrCKX1_concencus_sequence GCGGAWGAACATTTTCAACTTCTTTATCACGAMGGTGTTCCATACGTCGACGTTTCCGGAGGGGCATTATGGGAAGAAGTGTTGAAACGC
TD_CKX2_F1_R1              ----------------------------------------------------------------------------------------
TDCKX211R                 GCGGAWGAACATTTTCAACTTCTTTATCACGAMGGTGTTCCATACGTCGACGTTTCCGGAGGGGCATTATGGGAAGAAGTGTTGAAACGC
TDCKX211F                 SCKGAWGAACATTTTCAACTTCTTTATCACGAMGGTGTTCCATACGTCGACGTTTCCGGAGGGGCATTATGGGAAGAAGTGTTGAAACGC
TrCKX1_primers_F1_R1      ----------------------------------------------------------------------------------------
TrCKX111f(5)              GCKGAWGAACATTTTCAACTTCTTTATCACGAMGGTGTTCCATACGTCGACGTTTCCGGAGGGGCATTATGGGAAGASGT----------
TrCKX111r(6)              GCGGAWGAACATTTT----------------------------------------------------------------------------
TrCKX111F(1)              GSRGAWGAACATTTTYAACTTCTTTRTMRCGAAGGTGTTCCWTMCGTCGACGTTTCCGGAGGGGCATTATGGGAAGAAGTGTTGAAACGC
TrCKX111R(2)              GCGGAWGAACATTTTCAACTTCTTTATCACGAMGGTGTTCCATACGTCGACGTTTCCGGAGGGGCATTATGGGAAGAAGTGTTGAAACGM
TrCKX111F(3)              GCGGAWGAACATTTTCAACTTCTTTATCACGAMGGTGTTCCATACGTCGACGTTTCCGGAGGGGCATTATGGGAAGAAGTGTTGAAACGC
TrCKX111R(4)              GCGGAWGAACATTTTCAACTTCTTTATCACGAMGGTGTTCCATACGTCGACGTTTCCGGAGGGGCATTATGGGAAGAAGTGT-GAAACGM

T._repens_EF691439        TGCGTTTCGCAGTTTCAACTTGTTCCGAGATCGTGGACTGATTATCTCGGGTTA-------------------------------------
TrCKX1_concencus_sequence TGCGTTTCRCAGTTTCAACTTGTTCCGAGATCGTGGACTGATTATCTCGGGTTAACGGTCGGTGGAACGCTTTCTAACGCCGGTGTTAGT
TD_CKX2_F1_R1              ----------------------------------------------------------------------------------------
TDCKX211R                 TGCGTTTCRCAGTTTCAACTTGTTCCGAGATCGTGGACTGATTATCTCGGGTTAACGGTCGGTGGAACGCTTTCTAACGCCGGTGTTAGT
TDCKX211F                 TGCGTTTCRCAGTTTCAACTTGTTCCGAGATCGTGGACTGATTATCTCGGGTTAACGGTCGGTGGAACGCTTTCTAACGCCGGTGTTAGT
TrCKX1_primers_F1_R1      ---------------------------------cacctgactaatagagcccaa-------------------------------------
TrCKX111f(5)              ----------------------------------------------------------------------------------------
TrCKX111r(6)              ----------------------------------------------------------------------------------------
TrCKX111F(1)              TGCGTTTCGCARTTTCAACTTGTTCCGAGATCGTGGACTRG----------------------------------------------
TrCKX111R(2)              TGCGTT-CRCAGTT-CAACAG--TCCGAGATC-------------------------------------------------------
TrCKX111F(3)              TGCGTTTCRCAGTTTCAACTTGTTCCGAGATCGTGGACTGATTAATCTCGGGTT-------------------------------------
TrCKX111R(4)              TGCGTTTCRCAGTT-CAACAG--TCCGAGATTG-----------------------------------------------------
```

```
T._repens_EF691439        ----------------------------------------------------------------------------------------
TrCKX1_concencus_sequence GGTCAGACTTTCCGTTACGGTCCTCAAACGGCAAACGTAACGGAATTAGAAGTCGTTACTGGTAAAGGTGATACTTTAGTTTGTAATGAA
TD_CKX2_F1_R1              ----------------------------------------------------------------------------------------
TDCKX211R                 GGTCAGACTTTCCGTTACGGTCCTCAAACGGCAAACGTAACGGAATTAGAAGTCGTTACTGGTAAAGGTGATACTTTAGTTTGTAATGAA
TDCKX211F                 GGTCAGACTTTCCGTTACGGTCCTCAAACGGCAAACGTAACGGAATTAGAAGTCGTTACTGGTAAAGGTGATACTTTAGTTTGTAATGAA
TrCKX1_primers_F1_R1      ----------------------------------------------------------------------------------------
TrCKX111f(5)              ----------------------------------------------------------------------------------------
TrCKX111r(6)              ----------------------------------------------------------------------------------------
TrCKX111F(1)              ----------------------------------------------------------------------------------------
TrCKX111R(2)              ----------------------------------------------------------------------------------------
TrCKX111F(3)              ----------------------------------------------------------------------------------------
TrCKX111R(4)              ----------------------------------------------------------------------------------------

T._repens_EF691439        ----------------------------------------------------------------------------------------
TrCKX1_concencus_sequence AATCAAAATTCAGAACTTTTTTTCGCAACGCTTGGTGGTCTTGGTCAATTTGGTATCATCACTAGAGCCAGAATCGTTCTTCAACAAGCC
TD_CKX2_F1_R1              ----------------------------------------------------------------------------------------
TDCKX211R                 AATCAAAATTCAGAACTTTTTTTCGCAACGCTTGGTGGTCTTGGTCAATTTGGTATCATCACTAGAGCCAGAATCGTTCTTCAACAAGCC
TDCKX211F                 AATCAAAATTCAGAACTTTTTTTCGCAACGCTTGGTGGTCTTGGTCAATTTGGTATCATCACTAGAGCCAGAATCGTTCTTCAACAAGCC
TrCKX1_primers_F1_R1      ----------------------------------------------------------------------------------------
TrCKX111f(5)              ----------------------------------------------------------------------------------------
TrCKX111r(6)              ----------------------------------------------------------------------------------------
TrCKX111F(1)              ----------------------------------------------------------------------------------------
TrCKX111R(2)              ----------------------------------------------------------------------------------------
TrCKX111F(3)              ----------------------------------------------------------------------------------------
TrCKX111R(4)              ----------------------------------------------------------------------------------------

T._repens_EF691439        ----------------------------------------------------------------------------------------
TrCKX1_concencus_sequence CCTGATATGGTGAGGTGGATAAGGGTGATTTACTCGGAATTTGAGGATTTTACCAAAGATGCAGAGTGGCTGG??????????????????
TD_CKX2_F1_R1              ----------------------------------------------------------------------------------------
TDCKX211R                 CCTGATATGGTGAGGTGGATAAGGGTGATTTACTCGGAATTTGAGGATTTTACCAAAGATGCAGAGTGGCTGG-----------------
TDCKX211F                 CCTGATATGGTGAGGTGGATAAGGGTGATTTACTCGGAATTTGAGGATTTTACCAAAGATGCAGAGTGGCTGG-----------------
TrCKX1_primers_F1_R1      ----------------------------------------------------------------------------------------
TrCKX111f(5)              ----------------------------------------------------------------------------------------
TrCKX111r(6)              ----------------------------------------------------------------------------------------
TrCKX111F(1)              ----------------------------------------------------------------------------------------
TrCKX111R(2)              ----------------------------------------------------------------------------------------
TrCKX111F(3)              ----------------------------------------------------------------------------------------
TrCKX111R(4)              ----------------------------------------------------------------------------------------
```

```
T._repens_EF691439          ------------------------------------------------------
TrCKX1_concencus_sequence ?????????GATTACVT?GAAGGSTTCGTTGT----------------------
TD_CKX2_F1_R1               ---------CTAATGBA?CTTCCSAAACACTA----------------------
TDCKX211R                   ------------------------------------------------------
TDCKX211F                   ------------------------------------------------------
TrCKX1_primers_F1_R1        ------------------------------------------------------
TrCKX111f(5)                ------------------------------------------------------
TrCKX111r(6)                ------------------------------------------------------
TrCKX111F(1)                ------------------------------------------------------
TrCKX111R(2)                ------------------------------------------------------
TrCKX111F(3)                ------------------------------------------------------
TrCKX111R(4)                ------------------------------------------------------
```

6.4.8 TrCKX2

```
TrCKX2_CTR0036048420-cF2_2004072 TAAGAGTTGAGAAAGAGAATTAGAAAAAAAAAAAAAAAAACAACTATATATAACTCTCATAAACCAAATCAAGCCAATTTACTTAACTATG
TrCKX2F1                         ----------------------------------------------------------------------------------------------------
TrCKX2F2                         ----------------------------------------------------------------------------------------------------
TrCKX2R1                         ----------------------------------------------------------------------------------------------------
TrCKX2R2                         ----------------------------------------------------------------------------------------------------
ckx211_F1a                       ----------------------------------------------------------------------------------------------------
ckx211_R1a                       ----------------------------------------------------------------------------------------------------
ckx211_F1b                       ----------------------------------------------------------------------------------------------------
ckx211_R1b                       ----------------------------------------------------------------------------------------------------
ckx212_F1a                       ----------------------------------------------------------------------------------------------------
ckx212_R2a                       ----------------------------------------------------------------------------------------------------
ckx212_F1b                       ----------------------------------------------------------------------------------------------------
ckx212_R2b                       ----------------------------------------------------------------------------------------------------
ckx222_F2a                       ----------------------------------------------------------------------------------------------------
ckx222_R2                        ----------------------------------------------------------------------------------------------------
ckx222_F2b                       ----------------------------------------------------------------------------------------------------

TrCKX2_CTR0036048420-cF2_2004072 GCAAAGTATTTTATAGTTTCAAAAACCTTGATTATTCTACTATGTCTAATTAATAGTGTAGTGCACCCAATAGAAGCACTAACACAACCA
TrCKX2F1                         ----------------------------------------------------------CACCCAATAGAAGCACTAACACA----
TrCKX2F2                         ----------------------------------------------------------------------------------------------------
TrCKX2R1                         ----------------------------------------------------------------------------------------------------
TrCKX2R2                         ----------------------------------------------------------------------------------------------------
ckx211_F1a                       ----------------------------------------------------------------------------------------GACCC
ckx211_R1a                       ----------------------------------------------------------------------------------CSMTACACAACCA
ckx211_F1b                       ----------------------------------------------------------------------------------------GACCA
ckx211_R1b                       ----------------------------------------------------------TCCCCCCMAAGGAGAARSMTACACAACCA
ckx212_F1a                       ----------------------------------------------------------------------------------------AKCAT
ckx212_R2a                       ----------------------------------------------------------------------------------------------------
ckx212_F1b                       ----------------------------------------------------------------------------------------AAKCCA
ckx212_R2b                       ----------------------------------------------------------------------------ACTAACACAACCAT
ckx222_F2a                       ----------------------------------------------------------------------------------------------------
ckx222_R2                        ----------------------------------------------------------------------------------------------------
ckx222_F2b                       ----------------------------------------------------------------------------------------------------
```

```
TrCKX2_CTR0036048420-cF2_2004072 TGGTCATTATTAGATGCACCAAAAGATATCCTTCAAAACCTTATTCGTGACCCATTGAGCCTTTCACTAGCCTCAACCGATTTTGGTCAC
TrCKX2F1                         ----------------------------------------------------------------------------------------
TrCKX2F2                         ----------------------------------AAACCTTATTCGTGACCCATTG--------------------------------
TrCKX2R1                         ----------------------------------------------------------------------------------------
TrCKX2R2                         ----------------------------------------------------------------------------------------
ckx211_F1a                       TGGCTMTTATTAGATGCACCAAA-GATATCCTTCAAA-CCTTATTCGTGACCCMTTGAGCCTTTCACTAGCCTCAACCGATTTTGGTCAC
ckx211_R1a                       TGGTCATTATTAGATGCACCAAAAGAWATCCTTCAAAACCTTATTCGTGACCCMTTGAGCCTTTCACTMGCCTCAACCGATTTTGGTCAC
ckx211_F1b                       TGGTM-TTATTAGATGCACCAAA-GATATCCTTCAAA-CCTTATTCGTGACCCMTTGAGCCTTTCACTAGCCTCAACCGATTTTGGTCAC
ckx211_R1b                       TGGTCATTATTAGATGCACCAAAAGAWATCCTTCAAAACCTTATTCGTGACCCMTTGAGCCTTTCACTAGCCTCAACCGATTTTGGTCAC
ckx212_F1a                       GGCTMTT-MTTAGATGM-CCA-AAGATATYCTTCAAA-MCWTATTCGTGACCCCTTGAGCCTTTCACTAGCCTCAACCGATTTTGGTCAC
ckx212_R2a                       ----------------------------------------------------------------------------------------
ckx212_F1b                       TGGTMTT-ATTAGATGCACCATAAGATATCCTTCAAA-CCTTATTCGTGACCCMTTGAGCCTTTCACTAGCCTCAACCGATTTTGGTCAC
ckx212_R2b                       GGTCATT-ATTAGATGCACCAAAAGAWATCCTTCAAAACCTTATTCGTGACCCMTTGAGCCTTTCACTAGCCTCAACCGATTTTGGTCAC
ckx222_F2a                       ------------------------------------------------------------AARCTTTM-CTAGCCTCA-CCGATTTTGGT-MC
ckx222_R2                        ----------------------------------------------------------------------------------------
ckx222_F2b                       ------------------------------------------------------------CGCTTTM-CTAGCCTCA-CCGATTTTGGT-MC

TrCKX2_CTR0036048420-cF2_2004072 ATAATTCACAAAAACCCCGTCGCAATTTTTGCACCATCTTCCACAAATGACATAACAAAATTGATAAAATTCTCAAATTCCCTTCCTATC
TrCKX2F1                         ----------------------------------------------------------------------------------------
TrCKX2F2                         ----------------------------------------------------------------------------------------
TrCKX2R1                         ----------------------------------------------------------------------------------------
TrCKX2R2                         ----------------------------------------------------------------------------------------
ckx211_F1a                       ATAATTCACAAAAACCCMGTYGCAATTTTTGCACCATCTTCCACAAATGACATAWCAAAATTGATAAAATTCTCAAATTCCCTTCCTATC
ckx211_R1a                       ATAATTCACAAAAACCCMGTYGCAATTTTTGCACCATCTTCCACAAATGACATAWCAAAATTGATAAAATTCTCAAATTCCCTTCCTATC
ckx211_F1b                       ATAATTCACAAAAACCCMGTYGCAATTTTTGCACCATCTTCCACAAATGACATAWCAAAATTGATAAAATTCTCAAATTCCCTTCCTATC
ckx211_R1b                       ATAATTCACAAAAACCCMGTYGCAATTTTTGCACCATCTTCCACAAATGACATAWCAAAATTGATAAAATTCTCAAATTCCCTTCCTATC
ckx212_F1a                       ATAATTCACAAAAACCCAGTTGCA----TTATGCGGTGGTTCGAAG-----------------------------------------
ckx212_R2a                       ----------------------------CTCATTTCCATTTCACAAATGACATATCAAAATTGATAAAATTCTCAAATTCCCTTCCTATC
ckx212_F1b                       ATAATTCACAAAAACCCMGTYGCAATTTTTGCACCATCTTCCACAAATGACATAWCAAAATTGATAAAATTCTCAAATTCCCTTCCTATC
ckx212_R2b                       ATAATTCACAAAAACCCMGTYGCAATTTTTGCACCATCTTCCACAAATGACATAWCAAAATTGATAAAATTCTCAAATTCCCTTCCTATC
ckx222_F2a                       ATAATTCACAAAAACCCMGTYGCAATTTTTGCACCATCTTCCACAAATGACATAWCAAAATTGATAAAATTCTCAAATTCCCTTCCTATC
ckx222_R2                        --------------------------------TCAATTATTTCCAAATGTCTTCA----ATTGATAAAATTCTCAAATTCCCTTCCTATC
ckx222_F2b                       ATAATTCACAAAAACCCMGTYGCAATTTTTGCACCATCTTCCACAAATGACATAWCAAAATTGATAAAATTCTCAAATTCCCTTCCTATC
```

```
TrCKX2_CTR0036048420-cF2_2004072 CCTTTTACAATAGCTGCAAGAGGACAAGGACATTCAGTTAATGGACAATCCATGACAAATGATGGTGTTGTGTTGAACATGACTGAATTG
TrCKX2F1                         ----------------------------------------------------------------------------------------------------
TrCKX2F2                         ----------------------------------------------------------------------------------------------------
TrCKX2R1                         ----------------------------------------------------------------------------------------------------
TrCKX2R2                         ----------------------------------------------------------------------------------------------------
ckx211_F1a                       CCTTTTACAATAGCTGCAAGAGGACAAGGACATTCAGTTAATGGACAATCCATGACAAATGATGGTGTTGTGTTGAACATGACTGAATTG
ckx211_R1a                       CCTTTTACAATAGCTGCAAGAGGACAAGGACATTCAGTTAATGGACAATCCATGACAAATGATGGTGTTGTGTTGAACATGACTGAATTG
ckx211_F1b                       CCTTTTACAATAGCTGCAAGAGGACAAGGACATTCAGTTAATGGACAATCCATGACAAATGATGGTGTTGTGTTGAACATGACTGAATTG
ckx211_R1b                       CCTTTTACAATAGCTGCAAGAGGACAAGGACATTCAGTTAATGGACAATCCATGACAAATGATGGTGTTGTGTTGAACATGACTGAATTG
ckx212_F1a                       ----------------------------------------------------------------------------------------------------
ckx212_R2a                       CCTTTTACAATAGCTGCAAGAGGACAAGGACATTCAGTTAATGGACAATCCATGACAAATGATGGTGTTGTGTTGAACATGACTGAATTG
ckx212_F1b                       CCTTTTACAATAGCTGCAAGAGGACAAGGACATTCAGTTAATGGACAATCCATGACAAATGATGGTGTTGTGTTGAACATGACTGAATTG
ckx212_R2b                       CCTTTTACAATAGCTGCAAGAGGACAAGGACATTCAGTTAATGGACAATCCATGACAAATGATGGTGTTGTGTTGAACATGACTGAATTG
ckx222_F2a                       CCTTTTACAATAGCTGCAAGAGGACAAGGACATTCAGTTAATGGACAATCCATGACAAATGATGGTGTTGTGTTGAACATGACTGAATTG
ckx222_R2                        CCTTTTACAATAGCTGCAAGAGGACAAGGACATTCAGTTAATGGACAATCCATGACAAATGATGGTGTTGTGTTGAACATGACTGAATTG
ckx222_F2b                       CCTTTTACAATAGCTGCAAGAGGACAAGGA----------------------------------------------------------------

TrCKX2_CTR0036048420-cF2_2004072 AATAAAGGGATTGGAAATAATGGAAGTAGTAGAATTGTAGTGTTTGATAATTATGTAGATGTTGGTGGTGAACAAATTTGGATTGATGTT
TrCKX2F1                         ----------------------------------------------------------------------------------------------------
TrCKX2F2                         ----------------------------------------------------------------------------------------------------
TrCKX2R1                         ----------------------------------------------------------------------------------------------------
TrCKX2R2                         ----------------------------------------------------------------------------------------------------
ckx211_F1a                       AATAAAGGGWTTGGAAATAATGGRAGTAGTAGAATTGTAGTGTTTGATAATTATGTAGATGTTGGTGGTGAACAAATTTGGATTGATGTK
ckx211_R1a                       AATAAAGGGTTTGGAAATAATGGRAGTAGTAGAATTGTAGTGTTTGATAATTATGTAGATGTTGGTGGTGAACAAATTTGGATTGATGTK
ckx211_F1b                       AATAAAGGGWTTGGAAATAATGGRAGTAGTAGAATTGTAGTGTTTGATAATTATGTAGATGTTGGTGGTGAACAAATTTGGATTGATGTK
ckx211_R1b                       AATAAAGGGTTTGGAAATAATGGRAGTAGTAGAATTGTAGTGTTTGATAATTATGTAGATGTTGGTGGTGAACAAATTTGGATTGATGTK
ckx212_F1a                       ----------------------------------------------------------------------------------------------------
ckx212_R2a                       AATAAAGGGTTTGGAAATAATGGRAGTAGTAGAATTGTAGTGTTTGATAATTATGTAGATGTTGGTGGTGAACAAATTTGGATTGATGTK
ckx212_F1b                       AATAAAGGGWTTGGAAATAATGGRAGTAGTAGAATTGTAGTGTTTGATAATTATGTAGATGTTGGTGGTGAACAAATTTGGATTGATGTK
ckx212_R2b                       AATAAAGGGWTTGGAAATAATGGRAGTAGTAGAATTGTAGTGTTTGATAATTATGTAGATGTTGGTGGTGAACAAATTTGGATTGATGTK
ckx222_F2a                       AATAAAGGGWTTGGAAATAATGGRAGTAGTAGAATTGTAGTGTTTGATAATTATGTAGATGTTG----TGG-AAATTTTGGAAAAAGATT
ckx222_R2                        AATAAAGGGTTTGGAAATAATGGRAGTAGTAGAATTGTAGTGTTTGATAATTATGTAGATGTTGGTGGTGAACAAATTTGGATTGATGTK
ckx222_F2b                       ----------------------------------------------------------------------------------------------------
```

```
TrCKX2_CTR0036048420-cF2_2004072 TTGCATGCAAGCCTTGAAAAAGGACTTACACCACTTTCTTGGACTGATTATTTGTATTTATCGGTTGGTGGAACTCTCTATAATGCTGGT
TrCKX2F1                         ----------------------------------------------------------------------------------------
TrCKX2F2                         ----------------------------------------------------------------------------------------
TrCKX2R1                         ----------------------------------------------------------------------------------------
TrCKX2R2                         ------------------------------------------------------------------------------TGCTGGT
ckx211_F1a                       TTRCATGCAAGCCTTGAAAAAGGACTTACACCACTTTCTTGGACTGATTATTTGTATTTATCGGTTGGTGGAACTCTCTCTAATGCTGGT
ckx211_R1a                       TTRCATGCAAGCCTTGAAAAAGGACTTACACCACTTTCTTGGACTGATTATTTGTATTTATCGGTTGGTGGAACTCTCTCTAATGCTGGT
ckx211_F1b                       TTRCATGCAAGCCTTGAAAAAGGACTTACACCACTTTCTTGGACTGATTATTTGTATTTATCGGTTGGTGGAACTCTCTCTAATGCTGGT
ckx211_R1b                       TTRCATGCAAGCCTTGAAAAAGGACTTACACCACTTTCTTGGACTGATTATTTGTATTTATCGGTTGGTGGAACTCTCTCTAATGCTGGT
ckx212_F1a                       ----------------------------------------------------------------------------------------
ckx212_R2a                       TTRCATGCAAGCCTTGAAAAAGGACTTACACCACTTTCTTGGACTGAWTATTTGTATTTATCGGRTGGTGGAACTCTCTCTWAT------
ckx212_F1b                       TTRCATGCAAGCCTTGAAAAAGGACTTACACCACTTTCTTGGACTGATTATTTGTATTTATCGGTTGGTGGAACTCTCTCTAATGCTGGT
ckx212_R2b                       TTRCATGCAAGCCTTGAAAAAGGACTTACACCACTTTCTTGGACTGATTATTTGTATTTATCGGATGG-KGAACTCTCTCTTAC------
ckx222_F2a                       GAGGTATGGAGAAAAGGAAAAAA----------------------------------------------------------------
ckx222_R2                        TTRCATGCAAGCCTTGAAAAAGGACTTACACCACTTTCTTGGACTGATTATTTGTATTTATCGGTTGGTGGAACTCTCTCTAA-------
ckx222_F2b                       ----------------------------------------------------------------------------------------

TrCKX2_CTR0036048420-cF2_2004072 ATTAGTGGACAAACCTTTCGATTTGGTCCTCAAATTTCCAATGTCCTTGAATTGGATGTTGTTACGGGTATGTATATC
TrCKX2F1                         ---------------------------------------------------------------------------
TrCKX2F2                         ---------------------------------------------------------------------------
TrCKX2R1                         ----------------------------------------------------TGGATGTTGTTACGGGTATGTA----
TrCKX2R2                         ATTAGTGGACAAACC-------------------------------------------------------------
ckx211_F1a                       ATTAGTGGACAAACCTTTCGATTTGGTCCYCAAATTTCCAATGTCCTTGAATTGGATGTKTAMCCGGGKWATGTAA--
ckx211_R1a                       ATTAGTGGACAAACCTTTCGATT-GGTCCCCAAATT-CCAATGTCAT-GAACC-------------------------
ckx211_F1b                       ATTAGTGGACAAACCTTTCGATTTGGTCCYCAAATTTCCAATGTCCTTGAATTGGATGTTKTTACCSGGGTATGTA--
ckx211_R1b                       ATTAGTGGACAAACCTTTCGATT-GGTCCCTCAAATTCCAATGTCCT-GAATT-------------------------
ckx212_F1a                       ---------------------------------------------------------------------------
ckx212_R2a                       ---------------------------------------------------------------------------
ckx212_F1b                       AWRGTGGGACAAACC-------------------------------------------------------------
ckx212_R2b                       ---------------------------------------------------------------------------
ckx222_F2a                       ---------------------------------------------------------------------------
ckx222_R2                        ---------------------------------------------------------------------------
ckx222_F2b                       ---------------------------------------------------------------------------
```

6.4.9 TrCKX6 sequencing results

Sequencing data and PCR primers for TrCKX6 aligned to genomic and corresponding coding TrCKX6 sequence from PG with and TrCKX6 primers. Coding sequences shown in upper case

```
TrCKX_genomic --GTTGAAGGCAACATTAGAGTATGGGTTAGCACCTATGTCTTGG---ACTGATTAC---TTG---TATTTGTCTGTGGGTGGTACCCTATCCAATGCTGGTATTAGTGGCCAA
TrCKX6_CDS    --GTTGAAGGCAACATTAGAGTATGGGTTAGCACCTATGTCTTGG---ACTGATTAC---TTG---TATTTGTCTGTGGGTGGTACCCTATCCAATGCTGGTATTAGTGGCCAA
TrCKX6_F1     ----------------------TGGGTTAGCACCTATGTCTTGG-----------------------------------------------------------------------
TrCKX6_R2     ----------------------------------------------------------------------------------------------------------------
Ckx6_el_F1    ------------------------------------------------CAKTATTAC--CTTC---WTCTYGYTKTTGGGYGTT-YTCTTTCCATGYTGGAWTAAKTGGACCA-
Ckx6_ela_F1   ------------------------------------------------CAKGATTAC--STTG--CYTSYYGYYKKTGGKKRMM-YTSTKKCRAKKYKGKRRTAAGTGGACRW-
Ckx6_el_R2    --------------------CAYTGGGTTAGCACMTCTGTCTTGGTGTGAGAGGAATWCSTTC---MTMTGGGGCGAGGGGGTA-GTCTCATAACAKCGGAAAAAGRGGACAA-
Ckx6_sl_F1    ----------------------------------------------------CATTGTTAC---TTG---YGTTTGTCTGTGGGTGGY-CCCCTTTCCATGCTGKTCCA--KTGGMCA
Ckx6_sl_R2x   --------------------TGKTKGGKYAGCWCCTTGGGKKAGG---GCCTASGTG---TRG---GGGGGGAAGGGGGGGGGTYGMYTAATAASCGCCGGTAGTACMCCACAM
Ckx6_el_R2x   --------------------TGGTTGGG-CAGCACTGTTTTCTTGG---GCTGATTAG---TAT---TATTTTTTGGGGGGTGGCMTCCTCTACAATGGTGGTAGTAGSCGCCCC

TrCKX_genomic ACCTTCAATTATGGTCCTCAAATCTCCAATGTTTTTGAACTTGAT---GTTGTTACAggtaatttttcttatatatcaattatcatttgctcttgacatggtgtgtgtgtgtgt
TrCKX6_CDS    ACCTTCAATTATGGTCCTCAAATCTCCAATGTTTTTGAACTTGAT---GTTGTTACA------------------------------------------------------
TrCKX6_F1     ----------------------------------------------------------------------------------------------------------------
TrCKX6_R2     ----------------------------------------------------------------------------------------------------------------
Ckx6_el_F1    GCCTTCA-GCATGGACCCCMGATCAGTAACACCTTGCAGYTKGAW---GTWGTCACA------------------------------------------------------
Ckx6_ela_F1   GCCTTCAAGCATGGACCCCAGATCAGTAACACCTTGCAGTTGGAA---GA-GTCACA------------------------------------------------------
Ckx6_el_R2    RCCGTCAAGCARGGRTCCCAGARCAGTAACWCYTTRCA-RCGGAA---GA-GTCACA------------------------------------------------------
Ckx6_sl_F1    ACCTTCA-TTATGGTCCTCATWTCTCCATTGTCTTGAAAYTTGAT--KGTT------------------------------------------------------------
Ckx6_sl_R2x   TCCCAAAAGAAGCCTCCTMAAGTCAMCCAAKTTTTTCTMCATGTT---GTTGAAACTaatttttttttcttatataccaattatcatttkggwgstmcaaggtgtgagtgtgagk
Ckx6_el_R2x   TCCMWTWAKKKWSCTCCTATATTCAACAATGTTGAGAATCATGTT---GTTGCAWCTaggtaggtttcwtatatatcaattatcatttkctcgagscataw-gtswtcarcary

TrCKX_genomic gagtatgtgtactttactaattaattgtgtgaaata---------GGAAAGGGTGAGGTGATGACGTGTTCAGAAGAACGTAACTCAGACTTGTTCCATGCTGTTCTTGGTGGT
TrCKX6_CDS    ------------------------------------------GGAAAGGGTGAGGTGATGACGTGTTCAGAAGAACGTAACTCAGACTTGTTCCATGCTGTTCTTGGTGGT
TrCKX6_F1     ----------------------------------------------------------------------------------------------------------------
TrCKX6_R2     ------------------------------------------------------------------------------CATGCTGTTCTTGGTGGTC---------------
Ckx6_el_F1    ------------------------------------------GGAAAAGGAGAGGTGGTTACCTGCTCAGAGAACCGAGAAKCTGACC-TTTTTCATGCTGTTCTTGGTGG
Ckx6_ela_F1   ------------------------------------------GGAAAAGGAGAGGTG-------------------------------------------------------
Ckx6_el_R2    ------------------------------------------GGAAAAGGAGAGGTGRTACGKC--TCAGARRACCGAGAAGCAGACGRGTAG------------------
Ckx6_sl_F1    ----------------------------------------------------------------------------------------------------------------
Ckx6_sl_R2x   gaggagatgtactgaagaagaaaa---gaggaaaaa---------GGAAAGGGCGAGGGGATGACGRG-GCAGAAGAACGTAACTCAGAAGGGTAG------------------
Ckx6_el_R2x   gagtmwgtggacgtcawtaawwarawrya-gaaata---------GGAAAGGGYGAGGRGAWRACGGRMKCAGAAGAASRWWWYTYCMGRAMYTTRTMYG--------------

TrCKX_genomic CTTGGACAGTTTGGGATTATCACAAGAGCTAGAATTGCTCTCCAACCAGCTCCTCAAAGAGTATGTACTTCA
TrCKX6_CDS    CTTGGACAGTTTGGGATTATCACAAGAGCTAGAATTGCTCTCCAACCAGCTCCTCAAAGAGTATGTACTTCA
TrCKX6_F1     ------------------------------------------------------------------------
TrCKX6_R2     ------------------------------------------------------------------------
Ckx6_el_F1    TCTTG-------------------------------------------------------------------
```

6.4.10 TrCKX7 alignment of sequencing results

```
TrCKX7_pg TCCAGACAAGCCATTATTCTCATATTTTTAGTCCTTTTCTCCAAATTTTGTTGTTCCAGGTGGAACAGTAAAACATCATTGGTTACCCCAGAGGAAGATGTTTTCTACTTA
TrCKX7F1  ----------------------------------------------------------------------------TCATTGGTTACCCCAGAGGAAG--------------
TrCKX7F2  ------------------------------------------------------------------------------------------------------------------
TrCKX7R1  ------------------------------------------------------------------------------------------------------------------
TrCKX7R2  ------------------------------------------------------------------------------------------------------------------
ckx711F1  ------------------------------------------------------------------------------------------------CWGTTTTCTACYT
ckx712F1a ------------------------------------------------------------------------------------------------AGYTTYC--ACYT
ckx712F1b ----------------------------------------------------------------------------------------AAWRTTTTTCWACTWA
ckx712F1c ------------------------------------------------------------------------------------------------GATCKYCGTTCT
Ckx721F2a ------------------------------------------------------------------------------------------------------------
Ckx721F2b ------------------------------------------------------------------------------------------------------------
ckx711R1  ------------------------------------------------------------------------------------------------------------
Ckx721R1a ----------------------------------------------------------------------------------------------TGCATTCCTATCCTCT
Ckx721R1b ----------------------------------------------------------------------------------------------------------T
ckx712R2a ----------------------------------------------------------------------------GGTTACCCCAGAGGAAGATGTTTTCTACYTA
ckx712R2b ------------------------------------------------------------------------------------------------------------


TrCKX7_pg GTGGCATTCCTATCCTCTGCAGTCCCATCTTCAACTGGTGCAAACAGTTTAGAAAACATTCAAGCCCAAAACAAAAGGATTCTAGATTTTTGCACCAGTGCCCAACTAAAT
TrCKX7F1  ------------------------------------------------------------------------------------------------------------
TrCKX7F2  ---GCATTCCTATCCTCTGCAGTCC------------------------------------------------------------------------------------
TrCKX7R1  ------------------------------------------------------------------------------------------------------------
TrCKX7R2  ------------------------------------------------------------------------------------------------------------
ckx711F1  AGTGGMTTCCTATMCTMTGCAGTCCCATYTTCA-CTGGTGAAAACAGTTTAGAATACATTCTARMCCAAAACAAAAGGATYCTAGATTTCTGCACCAATGCCCAACTACAT
ckx712F1a AGTGRMTTCCTATCCKCTKCAKTCYCATYTTCA-CTGGTGMAAACAGTTTAGAAWACATTCWARMCCAAAACAAAAGGATYCTAGATTTYTGCACCARTGCCCAACTAMAT
ckx712F1b GTGGMATTCCWAWCCTCTGCAGTCYCATYTTCA-CTGGTGMAAACAGTTTAGAAWACATTCWARMCCAAAACAAAAGGATYCTAGATTTYTGCACCRRTGCCCAACTAMAT
ckx712F1c GGGTTACTWCMCTYYGYKKYCTTYWYTTSTTYA-CTGGKGYAAACAGTWTAGAAAACATTSAAGMCCAAAACAAAAGGATYCTAGATTTTTGCACCAATGCCCAACTAMAT
Ckx721F2a -------------------------CATCTTTCGCTGGTG--AACTGTTTAGAATG-ATTCYACCCTAACMMR---GGATYCTAKWTTTYTGCACCWSTGCCCAACTASRT
Ckx721F2b -------------------------CATCTTTCACTGGTGC-AACAGTTTAGAATACATTCTAGMCCAAAACAAAAGGATYCTAGATTTYTGCACCARTGCCCAACTACAT
ckx711R1  ----------------------------------------------------------------------------------TTTCTGCACCRATGCYCAACTACAT
Ckx721R1a GCAGCATTCATWTGCCTCGCRKTGACWTMKTCAMCWKGKGRAAMCAGKTTAGTMWACATTSTAAMCCAAAACAAAAGGATTCTAGATTTYTGCACCAATGCCCAASTACAT
Ckx721R1b TYTGCATTCCTATYCTCYGCAGTCCCATTTTCAACTKGKGAAAACAGTTTAGAAWACATTCWAAACCAAAACAAAAGGATYCTAGATTTYTGCACCMATGCYCAACTAMAT
ckx712R2a GTGGCATTCCTAWCMTCAGCAGTCWCATYTTCAACTGGKGMAAACMGTTTAGAAAACMTTCWARMCCAAAACAAAAGGATYCTAGATTTYTGCACCRRTGCYCAACTAMAT
ckx712R2b ------------------------------------------------------------------------AAACAAAAGGATTCTAGATTTTTGGMCCGRTGCTCACCTGCAT
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TrCKX7_pg GTGAAGCAGTATCTTCCACATTACAGCACACAGCAAGAATGGCAAGACCA-TTTTGGGTCAGATGGGAGAAATTGGTGGAAAGGAAAACAGCCTATGACCCACTGGCACTG
TrCKX7F1  ----------------------------------------------------------------------------------------------------------
TrCKX7F2  ----------------------------------------------------------------------------------------------------------
TrCKX7R1  ----------------------------------------------------------------------------------------------------------
TrCKX7R2  ----------------------------------------------------------------------------------GATACTGGGTGACCGTGAC
ckx711F1  GTGAAGCAATACCTTGCTCATTACAGCACACAGGAAGAATGGCAAACTCACTTTGGGTCAAAATGGGGGGCATTTGTGGAAAGAAAAAGAGTCTATGACCCACTAG-ATTA
ckx712F1a GTGAAGCARTATCTTSCWCATTACAGCACACAG-AAGAATGSCAAGAYC-TTTTG-----------------------------------------------------
ckx712F1b GTGAAGCARTAYCTTSCWCATTACAGCACACAGSAAGAATGSCAARMYCAYTTTGRGTCAAGWKGGRGRRMATKKGKGRAAAAGAAAAAAGYCTATGACMCACTGRCACTG
ckx712F1c GTGAAGCAACTTG-------------------------------------------------------------------------------------------
Ckx721F2a GTGAAGCARTAYCTTSCKCTTTACAGCACACAGSAAGAATGGCAARACAAYTTTGGGTCAAAATGGGRGAAATTTGKGAAAAGRAAAASAGYCTATGACCCACTRGCAYTR
Ckx721F2b GTGAAGCARTAYCTTSCTCATTACAGCACACAGSAAGAATGGCAARMYCAYTTTGRGTCAARAKGGRRRRCATKKGKGGAAAGAAAAAGAGYCTATGACCCACTRGCATTA
ckx711R1  GTGAAGCAATACCTTGCTCATTACAGCACACAGGAAGAATGGCAAACTCACTTTGGGTCAAAATGGGGGGCATTTGKGAAA-GAAAAASAGYCTAK-ACCCACTAGCACTR
Ckx721R1a GKGAAGCAGTATGATSCMCATTACAGCACACAGGAAGAATGGCAAGMCCAYTY-AGGTCAAGAAGGGAGAAA--GRGKGAAAGAAAARCAGTCAA-GACCCACAARCAMTA
Ckx721R1b GTGAAGCAATAYYTTKCTCATTACAGCACACAGSAAGAATGGCAARCYCAYTTTGGGTCAARATGGGRGRMA--TGGGGAAAGRAAAASAGCCTAK-ACCCACTAGCACTR
ckx712R2a GTGAAGCARTATYYTYCWCMTTACAGCACACAGSAAGAAWGGCAARMYC-AYTTGGGTCAAGATGGAGAAAC--TGGGGAAAGAAAACAKYG-------------------
ckx712R2b GTGGAGCAATTTCTTCMC-ATTACAGCTCRCAGGAAGRAWGGCAARSSCAYTT-GGGTCAAGATGG-AGAMA--TGGKGAAAGAAAA-CART-------------------
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TrCKX7_pg CTTGCCCCTGGCCATAGGATCTTTCAAAAGGCAATGTCTGCATCCTGT
TrCKX7F1  ------------------------------------------------
TrCKX7F2  ------------------------------------------------
TrCKX7R1  -----------GGTATCCTAGAAAGTTTTCCGA----------------
TrCKX7R2  GA----------------------------------------------
ckx711F1  CTTGCCCCTGGCCATAGAA-MTTTCAAAAGGAAAAAA-----------
ckx712F1a ------------------------------------------------
ckx712F1b CWAAAAA-----------------------------------------
ckx712F1c ------------------------------------------------
Ckx721F2a CTTGCCCCTGGCCATAGRACTTTTCAAAAGGCAATA------------
Ckx721F2b CTTGCCCCTGGCCATAGRATCTTTCAAAAGGMA---------------
ckx711R1  CT-GCRCCTG--------------------------------------
Ckx721R1a MG-GCACGAG--------------------------------------
Ckx721R1b CT-GCACCTGT--------------------------------------
ckx712R2a ------------------------------------------------
ckx712R2b ------------------------------------------------
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6.4.11 TrCKX3

>TrCKX3 peptide sequence
SPNQKPIISFLFSPLSTHTKKMAENYPFPIYFILLIITIPRLISTVGKTEQWKSTLPIELSTNKFISQKLKNDPKAIEKASSDYGNLVHDLPAAVFSPRTVNDIVSLIKLSFNSSVPFGIAARGQGHSTRGQAMAR-
DGVVVDMKGLKENKKNKNININIKVFEDSEVGVGGYYVDVGGEQLWIDVLYETLEYGLAPVSWTDYLYLTIGGTLSNAGISGQTFRYGPQITSVHQLDVVTGMHIFGFGFSKVIIL

>TrCKX3_cds_
TCACCAAACCAAAAACCCATTATTTCTTTTCTTTTTTCTCCTCTCTCTACACACACTAAAAAAATGGCTGAAAATTACCCTTTTCCAATATATTTCATTCTCCTAATAATAACCATACCACGTTTAATATCAACCGTAGGT
AAAACCGAACAATGGAAATCAACACTACCAATTGAATTATCAACAAACAAGTTCATATCTCAAAAACTCAAAAACGACCCAAAAGCCATTGAAAAAGCTTCAAGTGATTATGGCAACCTCGTACACGATTTACCAGCAGCG
GTATTTAGTCCAAGAACGGTAAACGACATAGTAAGTCTTATAAAGTTGTCGTTTAATAGTTCTGTCCCTTTTGGTATAGCCGCAAGAGGACAAGGACATTCCACGCGCGGACAAGCTATGGCACGT---
GATGGTGTCGTTGTTGATATGAAAGGATTGAAAGAAAATAAAAAAAATAAAAATATTAATATTAAGGTTTTTGAGGATTCTGAGGTTGGAGTTGGAGGATACTATGTTGATGTTGGAGGTGAACAATTATGGATTGATGTT
TTATATGAAACACTTGAATATGGACTTGCACCTGTTTCTTGGACTGATTATCTTTACTTAACTATTGGTGGAACATTATCTAATGCTGGTATTAGTGGTCAAACATTTCGTTATGGTCCTCAAATTACTAGTGTTCATCAA
TTGGATGTTGTCACAGGTATGCACATTTTCGGATTTGGATTTTCTAAAGTCATAATTTTATAA

6.4.12 TrCKX4

>TrCKX4 peptide sequence
TTSPGFYYEKNVSFIEFLNRVRSGELKLQSQGLWDVPHPWLNMFIPKSRIMDFNSGVFKKIIQKRNITTGPVLVYPMNRNKYALNFFRWDNNMSATIPDDEDDVFYAVGFLHSSGFDNWKAFDAQNKEILQFCNDAEIKYK
LYLPHYNTQEEWTNHFGPKKWKTFLQRKYEFDPR

>TrCKX4_cds in upper case
ctataaattaaagaaaaaatatgtataattaaaaaatgtaataaatattaaactaattaatttttttaatgtgtgtaggaaatccaagttttgctcccaggactgaACTACATCCCCTGGATTTTATTATGAAAAAAATGT
GTCATTTATTGAGTTCTTAAATAGAGTAAGAAGTGGAGAGTTGAAACTACAATCACAAGGATTATGGGATGTTCCTCATCCATGGCTTAATATGTTTATACCAAAATCAAGAATCATGGATTTTAATTCAGGTGTCTTCAA
AAAGATAATTCAAAAAAGAAACATCACCACAGGACCTGTCTTGGTTTATCCCATGAATAGAAATAAGTATGCATTGAAT---
taaccttgttgacatgatttttcttaagatttgtttctcttttttttttttttattaagagaatttacattcacttttttatgatgtccaaataataatatattaaaatacacataaaaaagtatatattaaaaagtatg
attttatgatgtgcaattacacatgttaaacaaatctttgaaatattcatgatacacttgtaaaatacagtagttattagtgtctctttgatcaatatataaaaaatcctcatttcccatttttatgaaaaaaaaatacat
gtatgataataatttttggtctaatgtcgatgaatttttttaaattagatacaataaaaaaaaataatgaccaaatagttaaaatatatgtatattttaacttgaaatgtcacaagacacccttttatagttgacaccagatgt
gaaaaatttacggtatcggtgcatatgaattaaattatttaaacttgaaatgtatgtaagaagagataagtacaagtttctcttttatatacatccaattcattcatttatttttttcattaaatttgtaaaaataaaatc
taatttataggaattaattaa---
TTTTTCAGGTGGGACAATAACATGTCAGCAACAATACCAGATGATGAGGATGATGTCTTCTATGCTGTTGGATTTTTGCACTCAAGTGGGTTTGATAATTGGAAAGCATTTGATGCTCAAAACAAAGAAATTTTGCAATTT
TGTAATGATGCTGAAATTAAGTATAAGCTATATCTTCCCCACTACAACACACAAGAAGAGTGGACAAACCATTTTGGTCCTAAAAAATGGAAAACTTTTTTACAAAGAAAATATGAGTTTGATCCAAGA

6.4.13 TrCKX5

>TrCKX5 peptide sequence
GTLSNGGISGMTFRYGPQVTNVHELDVITGLIH

>TrCKX5_cds
AGGAACACTTTCCAATGGTGGAATTAGTGGCATGACGTTTCGATATGGACCTCAAGTCACAAACGTTCATGAATTGGATGTGATTACAGGTTTGATTCAT

6.4.14 CKX peptide alignment

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TrCKX1  ---------- ---------- ---------- ---------- ---------- ---------- -----FVHGN DTEST----- ---------- --------PN DDVSS---?Q
TrCKX2  ---------- ---------* ELRKRIRKKK KKQLYITLIN QIKPIYLTMA KYFIVSKTLI ILLCLIN--- SVVHP----- IEALTQPWSL LD-----APK DILQN-----
TrCKX3  ---------- ---------- ---SPNQKPI ISFLFSP--- --LSTHTKKM -AENYPFPIY FILLIIT--I PRLIS----- TVGKTEQWKS TLPIELSTNK FISQK-----
TrCKX4  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX5  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX6  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX7  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
AtCKX1  ---------- ---SSTLNLK FTSTSLIPFR LLFFAISLNK VEMGLTS--S LRFHRQNNKT FLGIFMILVL SCIPGRTNLC SNHSVSTPKE LPSSNPSDIR SSLVSLDLEG
AtCKX2  ---------- ---------- ---------- ------VVIL FFKNQIR--- EREKHKQMAN -LRL-MITLI TVLM------ ---------- --ITKSSNGI KIDLPKSLNL
AtCKX3  ---------- ---------- ---------- -------KTY FSKKMAS--Y NLRSQVRLIA ITIVIIITLS TPIT------ ---------- --TNTSPQPW NILSHNEFAG
AtCKX4  ---------- ---------- ---------* SHNQQDTLIV ISQPPSRFPI NLPVC*IMTN TLCLSLITLI TLFISLT--- ---------- PTLIKSDEGI DVFLPISLNL
AtCKX5  ---------- ---------- ---------- ---------- ---------- ---MNREMTS SFLLLTFAIC KLIIA----- ---------- VGLNVGPSEL LRIGAIDVDG
AtCKX6  -SPFGDWLPR PFRNHTTFLF IYL*DQKPFS ILGFYTLVSQ KSRGLMSYLH ASLLRKRTML IVRSFTILLL SCIAF----- ---------- KLACCFSSSI SSLKALPLVG
AtCKX7  ---------- ---------Q TNKKRFGSKS *KASIYIRVS LFSIYTQSHT HTHTHTKMIA YIEPYFLEND AEAAS----- ---------- -AATAAGKST DGVSESLNIQ
OsCKX1  ---------- ---------- ---------- ---------- ---------- ---MAARCSI A-FMVMASCL SVV------- ---------- --VSGGLPG- -DLFAHSVAS
OsCKX2  ---------- ---------- ---------- ---------- ---------- ---MAVLLML NCFVKATAPP PWPP------ ---------- SASSASFLD- -DLGDLGIAP
OsCKX3  ---------- ---------- ---------- ---------- ---------- --MEVAMVCT RVNLLILILS LCSP------ ---------- ---------Y KFIQSPMD-F
OsCKX4  ---------- ---------- ---------- ---------- ---------M RGAMKPSIVH CLKLLMLLAL GGVT------ ---------- MHVPDEDDVV ASLGALRLDG
OsCKX5  ---------- ---------- ---------- ---------- ---------- -------MAW CLVFMVFLIY CLIST----- ---------- VGLPVAPADE AAMQLGGVGG
OsCKX6  ---------- ---------- ---------- ---------- ---------- ---MAARCSI A-FMVMASCL SVV------- ---------- --VSGGLPG- -DLFAHSVAS
OsCKX7  ---------- ---------- ---------- ---------- ---------- ---MAARCSI A-FMIMASCL SVV------- ---------- --VSGGLPG- -DLFALSVAS
OsCKX8  ---------- ---------- ---------- ---------- ---------- ----MELKAM YLYAAVLAVL LCSS------ ---------- ---------V NFIQSPTDVL
OsCKX9  ---------- ---------- ---------- ---------- ---------- ---MRPSLLQ YLKLLLLLAL GGVTT----- ---------- MHVPKQD-VP SSLEELTLDG
OsCKX10 ---------- ---------- ---------- ---------- ---------- ---MMPRAQL TTFLIVTSFL STVPYLR--- ---------- APVHGGVLTS YDVSSLDIMS
OsCKX11 ---------- ---------- ---------- ---------- ---------- ------MMLA YMDHAAAAAE PDAG------ ---------- ---------- --------AE
PsCKX1  ---------- ---------- ---------- ---------- ---------- -------MIA YLEHFVHGND TESS------ ---------- --------PN DDVSS---LQ
RfCKX1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------MS
PvCKX   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------P
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TrCKX1  G--SFHFSPI SIATKDFGGM KSS------- -TPLA?IRPY ?TSDVARAVK AATTTT---- -------NLT VAPRGNGHSI NGQAMA-EKG LVLDMRATA? EHFQLLYH?G
TrCKX2  --LIRDPLSL SLASTDFGHI IH-------- KNPVAIFAPS STNDITKLIK FSNSLP---- ------IPFT IAARGQGHSV NGQSMTN-DG VVLNMTELNK GIGNNGSS--
TrCKX3  --LKNDPKAI EKASSDYGNL VH-------- DLPAAVFSPR TVNDIVSLIK LSFNSS---- ------VPFG IAARGQGHST RGQAMAR-DG VVVDMKGLKE NKKNKNINIK
TrCKX4  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX5  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX6  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX7  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
AtCKX1  -YISFD--DV HNVAKDFGNR YQ-------- LPPLAILHPR SVFDISSMMK HIVHLGST-- ------SNLT VAARGHGHSL QGQALAHQ-G VVIKMESL-- RSP---DIRI
AtCKX2  -TLSTDPSII SAASHDFGNI TTV------- -TPGGVICPS STADISRLLQ YAANGK---- ------STFQ VAARGQGHSL NGQASVSG-G VIVNMTCIT- -------DVV
AtCKX3  -KLTSSSSSV ESAATDFGHV TKI------- -FPSAVLIPS SVEDITDLIK LSFDSQ---- ------LSFP LAARGHGHSH RGQASAKD-G VVVNMRSMVN RDR----GIK
AtCKX4  -TVLTDPFSI SAASHDFGNI TDE------- -NPGAVLCPS STTEVARLLR FANGGFSYNK GSTSPASTFK VAARGQGHSL RGQASAPG-G VVVNMTCLAM AAKP--AAVV
AtCKX5  -HFTVHPSDL ASVSSDFGML KSP------- EEPLAVLHPS SAEDVARLVR TAY--GSA-- ------TAFP VSARGHGHSI NGQAAAGRNG VVVEMNHG-- --VTGTPKPL
AtCKX6  -HLEFE--HV HHASKDFGNR YQ-------- LIPLAVLHPK SVSDIASTIR HIWMMGTH-- ------SQLT VAARGRGHSL QGQAQTRH-G IVIHMESL-- HPQ---KLQV
AtCKX7  GEILCGGAAA DIAGRDFGGM NCV------- -KPLAVVRPV GPEDIA---G AVKAALRS-- ------DKLT VAARGNGHSI NGQAMAEG-G LVVDMSTTAE NHFEVGYLSG
OsCKX1  -KLRVDRDTT ARASSDFGRI VAA------- -APEAVLHPA TPAEIAELVR FSASSP---- ------SPFP VAPRGQGHSA RGQSLAPG-G VVVDMRALAA RRGRVNVSAG
OsCKX2  -LIRADEAGT ARASADFGNL SVAGVGAPRL AAAAAVLYPS RPADIAALLR ASCARP---- ------APFA VSARGCGHSV HGQASAPD-G VVVDMASLGR LQGGGARRLA
OsCKX3  GPLNLL-PTT TTASSDFGRI LFH------- -SPSAVLKPQ APRDISLLLS FLS-ASPL-- ------GKVT VAARGAGHSI HGQAQALD-G IVVEMSSLPS EIEFY--RRG
OsCKX4  -HFSFD--DA HAAARDFGNR CS-------- LLPAAVLHPG SVSDVAATVR RVFQLGRS-- ------SPLT VAARGHGHSL LGQSQAAG-G IVVKMESL-- AAAAARAVRV
OsCKX5  GRLSVEPSDV MEASLDFGRL TS-------- AEPLAVFHPR GAGDVAALVK AAY--GSA-- ------SGIR VSARGHGHSI SGQAQAAG-G VVVDMSHGWR AEAAERTLPV
OsCKX6  -KLRVDRDTT ARASSDFGRI VAA------- -APEAVLHPA TPAEIAELVR FSASSP---- ------SPFP VAPRGQGHSA RGQSLAPG-G VVVDMRALAA RRGRVNVSAG
OsCKX7  -KLRVDRNST ARASSDFGRI VAA------- -APEAVLHPA TPAEIAELVR FSASSP---- ------SPFP VAPRGQGHSA RGQSLAPG-G VVVDMRALAS RRGRVNVSAG
OsCKX8  GPVALL-EPT PSSARDFGAV VSD------- -APFAVMRPE SPDDIALLLG ALSSTAPS-- ------PRAT VAAVGAGHSL HGQAQARD-G IVVETRALPR DVHVVSARAH
OsCKX9  -HFSFH--DV SAAAQDFGNL SS-------- FPPVAVLHPG SVADIATTIR HVFLMGEH-- ------STLT VAARGHGHSL YGQSQAAE-G IIISMESL-- QSN---TMRV
OsCKX10 -KIHTDHDAT TKASSDFGHI VHA------- -TPNGVFRPT FPADIAALIR LSLSQP---- ------TPFT VAPRGKGHSS RGQAFAPG-G IVVDMSALGD HGHHTSHRID
OsCKX11 PAVAAVDAAE FAAAMDFGGL VSA------- -RPAAVVRPA SSDDVA---S AIRAAART-- ------AHLT VAARGNGHSV AGQAMARG-G LVLDMRALPR RMQLVVAPSG
PsCKX1  SSFHF--APN SIATKDFGGL KSS------- -NPLAVIRPY STADVA---R AVKAAATT-- ------TNLT VAARGNGHSI NGQAMAEK-G LVLDMRATAE EHFQLLYLEG
RfCKX1  GIWHTDDVHL TSAGADFGNC IHA------- -KPPVVVVPR TVADVQEALR YTAARN---- --------LS LAVRGSGHST YGQCQADG-G VVLDMKRFN- --------TV
PvCKX   -QILS----- -LASTDYGQI VQK------- -TPLEVFEPS SVSDISALIN FSNSLP---- ------TPFT IAPRGKAHSI LGQALTGN-G VVLNMTNLNG SLIFVSKCDG
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TrCKX1  ---------- VPYVDVSGGA LWEEVLKRCV ?QFQLVPRSW TDYLGLTVGG TLSNAGVSGQ TFRYGPQTAN VTELEVVTGK GDTLVCNENQ NSELFFATLG GLGQFGIITR
TrCKX2  -----RIVVF DNYVDVGGEQ IWIDVLHASL EKG-LTPLSW TDYLYLSVGG TLYNAGISGQ TFRFGPQISN VLELDVVTG- ---------- ---------- ----------
TrCKX3  VFEDSEVGVG GYYVDVGGEQ LWIDVLYETL EYG-LAPVSW TDYLYLTIGG TLSNAGISGQ TFRYGPQITS VHQLDVVTG- ---------- ---------- ----------
TrCKX4  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX5  ---------- ---------- ---------- ---------- ---------G TLSNGGISGM TFRYGPQVTN VHELDVITGL ---------- ---------- ----------
TrCKX6  ---------- ---------- -----LKATL EYG-LAPMSW TDYLYLSVGG TLSNAGISGQ TFNYGPQISN VFELDVVTGK GEVMTCSEER NSDLFHAVLG GLGQFGIITR
TrCKX7  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
AtCKX1  YKGKQ----- -PYVDVSGGE IWINILRETL KYG-LSPKSW TDYLHLTVGG TLSNAGISGQ AFKHGPQINN VYQLEIVTGK GEVVTCSEKR NSELFFSVLG GLGQFGIITR
AtCKX2  VSKDK----- -KYADVAAGT LWVDVLKKTA EKG-VSPVSW TDYLHITVGG TLSNGGIGGQ VFRNGPLVSN VLELDVITGK GEMLTCSRQL NPELFYGVLG GLGQFGIITR
AtCKX3  VSRTC----- -LYVDVDAAW LWIEVLNKTL ELG-LTPVSW TDYLYLTVGG TLSNGGISGQ TFRYGPQITN VLEMDVITGK GEIATCSKDM NSDLFFAVLG GLGQFGIITR
AtCKX4  ISADG----- -TYADVAAGT MWVDVLKAAV DRG-VSPVTW TDYLYLSVGG TLSNAGIGGQ TFRHGPQISN VHELDVITGK GEMMTCSPKL NPELFYGVLG GLGQFGIITR
AtCKX5  VRPDE----- -MYVDVWGGE LWVDVLKKTL EHG-LAPKSW TDYLYLTVGG TLSNAGISGQ AFHHGPQISN VLELDVVTGK GEVMRCSEEE NTRLFHGVLG GLGQFGIITR
AtCKX6  YSVDSP---- APYVDVSGGE LWINILHETL KYG-LAPKSW TDYLHLTVGG TLSNAGISGQ AFRHGPQISN VHQLEIVTGK GEILNCTKRQ NSDLFNGVLG GLGQFGIITR
AtCKX7  GDA------- TAFVDVSGGA LWEDVLKRCV SEYGLAPRSW TDYLGLTVGG TLSNAGVSGQ AFRYGPQTSN VTELDVVTGN GDVVTCSEIE NSELFFSVLG GLGQFGIITR
OsCKX1  GAGAA----- -PYVDAGGEQ LWADVLRATL EHG-LAPRVW TDYLRITVAG TLSNAGIGGQ AFRHGPQIAN VLELDVITGR GDMVTCSRDK EPDLFFAVLG GLGQFGIITR
OsCKX2  VSVEG----- -RYVDAGGEQ LWVDVLRASM AHG-LTPVSW TDYLHLTVGG TLSNAGISGQ AFRHGPQISN VLELDVITGV GEMVTCSKEK APDLFDAVLG GLGQFGVITR
OsCKX3  EGD------- VSYADVGGGI MWIELLEQSL -KLGLAPRSW TDYLYLTIGG TLSNAGISGQ TFKHGPQISN VLQLEVVTGR GEIVTCSPTK DAELFNAVLG GLGQFGIITR
OsCKX4  HGGAS----- -PHVDAPGGE LWINVLHETL KHG-LAPRSW TDYLHLTVGG TLSNAGVSGQ AFRHGPQVSN VNQLEIVTGR GEVVTCSHEV NSDLFYAALG GLGQFGIITR
OsCKX5  YSPALG---- GHYIDVWGGE LWIDVLNWTL AHGGLAPRSW TDYLYLSVGG TLSNAGISGQ AFHHGPQISN VYELDVVTGK GEVVTCSESN NPDLFFGALG GLGQLGIITR
OsCKX6  GAGAA----- -PYVDAGGEQ LWADVLRATL EHG-LAPRVW TDYLRITVAG TLSNAGIGGQ AFRHGPQIAN VLELDVITGR GDMVTCSRDK EPDLFFAVLG GLGQFGIITR
OsCKX7  ---AA----- -PYVDAGGEQ LWADVLRATL EHG-LAPRVW TDYLRITVAG TLSNAGIGGQ AFRHGPQIAN VLELDVITGT GDMVTCSRDK DSDLFFAVLG GLGQFGIITR
OsCKX8  GGDDDATV-- RAYADVGAGA LWVEVLEECL -KLGLAPPSW TDYLYLTVGG TLSNGGISGQ TFKHGPQISN VLQLEVVTGK GEVVTCSPTE IPELFFAVLG GLGQFGIITR
OsCKX9  NPGVS----- -PYVDASGGE LWINVLHETL KYG-LAPKSW TDYLHLTVGG TLSNAGVSGQ TFRHGPQISN VNELEIVTGR GDVITCSPEQ NSDLFHAALG GLGQFGVITR
OsCKX10 VSVDR----- -MYVDAGGEQ LWIDVLHTAL KHG-LTPRVW TDYLRITVGG TLSNAGIGGQ AFRHGPQISN VHELDVVTGM GEMITCSPEV NSALFFAVLG GLGQFGVITR
OsCKX11 ---------- EKFADVPGGA LWEEVLHWAV SKHGLAPASW TDYLRLTVGG TLSNGGVSGQ SFRYGPQVSN VAQLEVVTGD GECHVCSRSA DPDLFFAVLG GLGQFGVITR
PsCKX1  ---------- LPYVDVSGGA LWEEVLKRCV SQFQLVPRSW TDYLGLTVGG TLSNAGVSGQ TFRYGPQTAN VTELEVVTGK GESLVCSENQ NSELFFATLG GLGQFGIITR
RfCKX1  HDVRS----- -GQATIDAGV RWSDVVAATL SRQ-QTPPVL TDYLGTTVGG TLSVGGFGGS SHGFGLQTDN VDSLAVVTGS GDFRECSAVS NSELFDAVRG GLGQFGVIVN
PvCKX   ENPLN----- -CYADVGGEV VWIDVLHATL ERG-LTPLSW TDYLYLTVGG TLSNAGISGQ AFRFGPQISN VLELDVVTGK GDLVSCSREK ESELFYGVLG GLGQFGVITR
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TrCKX1  ARIVLQQAPD MVRWIRVIYS EFEDFTKDAE WL-------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX3  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX4  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX5  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX6  ARIALQPAPQ RV-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX7  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
AtCKX1  ARISLEPAPH MVKWIRVLYS DFSAFSRDQE YLISKE---- -----KTFDY VEGFVIINRT DLLNN----- --------WR SS-FSPNDST QASRFKSDGK --TLYCLEVV
AtCKX2  ARIVLDHAPK RAKWFRMLYS DFTTFTKDQE RLIS--MAND IG-----VDY LEGQIFLS-N GVVD------ ---------- TSFFPPSDQS KVADLVKQHG --IIYVLEVA
AtCKX3  ARIKLEVAPK RAKWLRFLYI DFSEFTRDQE RVIS--KTD- -G-----VDF LEGSIMVD-H GPPDN----- --------WR STYYPPSDHL RIASMVKRHR --VIYCLEVV
AtCKX4  ARIALDHAPT RVKWSRILYS DFSAFKRDQE RLIS--MTND LG-----VDF LEGQLMMS-N GFVD------ ---------- TSFFPLSDQT RVASLVNDHR --IIYVLEVA
AtCKX5  ARISLEPAPQ RVRWIRVLYS SFKVFTEDQE YLIS-MHGQ- -----LKFDY VEGFVIVD-E GLVNN----- --------WR SSFFSPRNPV KISSVSSNGS --VLYCLEIT
AtCKX6  ARIALEPAPT MVKWIRVLYL DFAAFAKDQE QLISAQG--- -----HKFDY IEGFVIINRT GLLNS----- --------WR LS-FTAEEPL EASQFKFDGR --TLYCLELA
AtCKX7  ARVLLQPAPD MVRWIRVVYT EFDEFTQDAE WLVSQKNE-- -----SSFDY VEGFVFVNGA DPVNG----- --------WP TVPLHPDHEF DPTRLPQSCG -SVLYCLELG
OsCKX1  ARIGLEPAPK RVRWVRLAYS DVVTFTRDQE LLIS-KRASE AG-----FDY VEGQVQLN-R TLTE------ ------GPKS TPFFSRFDID RLAGLASESV SGVIYFIEGA
OsCKX2  ARIPLAPAPA RARWVRFVYT TAAAMTADQE RLIAVDRAGG AGAVGGLMDY VEGSVHLN-Q GLVETWRTQP QPPSPSSSSS SSFFSDADEA RVAALAKEAG -GVLYFLEGA
OsCKX3  ARILLQEAPQ KVKWVRAFYD DFATFTKDQE LLVSMP---- -----VLVDY VEGFIVLNEQ SLHS------ ---------S SIAFPTNVDF NPDFGTKNNP -KIYYCIEFA
OsCKX4  ARIALEPAPK MVRWIRVLYS DFETFTEDQE KLIASE---- -----KTFDY IEGFVIINRT GILNN----- --------WR TS-FKPQDPV QASQFQSDGR --VLYCLELT
OsCKX5  ARIALEPAPH RVRWIRALYS NFTEFTADQE RLISLQHGG- -----RRFDY VEGFVVAA-E GLINN----- --------WR SSFFSPQNPV KLSSLKHHSG --VLYCLEVT
OsCKX6  ARIGLEPAPK RVRWVRLAYS DVVTFTRDQE LLIS-KRASE AG-----FDY VEGQVQLN-R TLTE------ ------GPKS TPFFSRFDID RLAGLASESV SGVIYFIEGA
OsCKX7  ARIGLMPAPK RVRWVRLAYS DVATFTKDQE LLIS-KRASE AG-----FDY VEGQVQLN-R TLTE------ ------GPKS TPFFSSSDIG RLAGLASKSV SGVIYVIEGT
OsCKX8  ARIPLQLAPP KVRWVRAFYD SFETFTGDQE LLVSMP---- -----EQVDY VEGFMVLNEQ SLHS------ ---------S SVAFPAQLNF SPDFGSKGRK -KVYYCIEFA
OsCKX9  ARIPLEPAPK MVRWLRVLYL DFTSFTEDQE MLISAE---- -----KTFDY IEGFVIINRT GILNN----- --------WR SS-FNPQDPV RSSQFESDGK --VLFCLEMT
OsCKX10 ARIRLEPAPK RVKWVRIAYS DVHPFTTDQE LLIS-KWASG SG-----FDY VEGQVQLN-R TLTQ------ ------GRRS SSFFSATDLA RLTGLAIDTG SVAIYYIEGA
OsCKX11 ARIPLSPAPQ TVRWTRVVYA SFADYAADAE WLVTRPPH-- -----EAFDY VEGFAFVRSD DPVNG----- --------WP TVPIPDGAHF DASLLPANAG -PVLYCLEVA
PsCKX1  ARIILQQAPD MVRWIRVIYS EFEEFTKDAE WLVTLPEG-- -----DGFDY VEGFVVANND DPCNG----- --------WP TIPMGSNQIF DPVHLSPSAG -PVLYCLELA
RfCKX1  ATIRLTAAHE SVRQYKLQYS NLGVFLGDQL RAMSNR---- ------LFDH VQGRIRVDAD GHLR------ ---------- ---------- ---------- ----YRLDLA
PvCKX   ARILLGPAPT ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
```

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TrCKX1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX3  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX4  ---------- ---------- ---------- --PGFYYEKN VSFIEFLNRV RSGELKLQSQ GLWDVPHPWL NMFIPKSRIM DFNSGVFKKI IQKRNITTGP VLVYPMNRNK
TrCKX5  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX6  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX7  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------SR
AtCKX1  KYFN---PEE AS----SMDQ ETGKLLSELN YIPSTLFSSE VPYIEFLDRV HIAERKLRAK GLWEVPHPWL NLLIPKSSIY QFATEVFNNI LTSNN--NGP ILIYPVNQSK
AtCKX2  KYYD---DPN LP----IISK VIDTLTKTLS YLPGFISMHD VAYFDFLNRV HVEENKLRSL GLWELPHPWL NLYVPKSRIL DFHNGVVKDI LLKQKSASGL ALLYPTNRNK
AtCKX3  KYYD---ETS QY----TVNE EMEELSDSLN HVRGFMYEKD VTYMDFLNRV RTGELNLKSK GQWDVPHPWL NLFVPKTQIS KFDDGVFKGI ILRNNITSGP VLVYPMNRNK
AtCKX4  KYYD---RTT LP----IIDQ VIDTLSRTLG FAPGFMFVQD VPYFDFLNRV RNEEDKLRSL GLWEVPHPWL NIFVPGSRIQ DFHDGVINGL LLNQTSTSGV TLFYPTNRNK
AtCKX5  KNYH---DSD SE----IVDQ EVEILMKKLN FIPTSVFTTD LQYVDFLDRV HKAELKLRSK NLWEVPHPWL NLFVPKSRIS DFDKGVFKGI L-GNKTSG-P ILIYPMNKDK
AtCKX6  KYLK---QDN KD----VINQ EVKETLSELS YVTSTLFTTE VAYEAFLDRV HVSEVKLRSK GQWEVPHPWL NLLVPRSKIN EFARGVFGNI LTDTS--NGP VIVYPVNKSK
AtCKX7  LHYR---DSD SN---STIDK RVERLIGRLR FNEGLRFEVD LPYVDFLLRV KRSEEIAKEN GTWETPHPWL NLFVSKRDIG DFNRTVFKEL VKNG--VNGP MLVYPLLRSR
OsCKX1  MYYN---EST TA----SVDQ KLTSVLEQLS FDKGFVFTKD VSYVQFLDRV REEERILRSI GMWDVPHPWL NLFVPQSRIL DFDTGVLKGV FVGAN-PVGV ILMYPMNRNM
OsCKX2  IYFGGAAGPS AA----DVDK RMDVLRRELR HERGFVFAQD VAYAGFLDRV HDGELKLRAA GLWDVPHPWL NLFLPRSGVL AFADGVFHGI LSRTP-AMGP VLIYPMNRNK
OsCKX3  VHDY---QNK NI----NVEQ VVEVISRQMS HIASHLYSVE VSYFDFLNRV RMEEMSLRNS GLWEVHHPWL NMFVPSAGIS DFRDLLMDSI SPDN--FEGL ILIYPLLRHK
OsCKX4  MNFN---HDE AD----IMEQ EVGALLSRLR YISSTLFYTD VTYLEFLDRV HTSELKLRAQ GLWEVPHPWL NLLIPRSTVH KFAKEVFGKI LKDSN--NGP ILLYPVNRTK
OsCKX5  KNYD---DST AV----TVDQ DVEALLGELN FIPGTVFTTD LPYVDFLDRV HKAELKLRGK GMWEVPHPWL NLFVPASRIA DFDRGVFRGV L-GSRTAGGP ILIYPMNRHK
OsCKX6  MYYN---EST TA----SVDQ KLTSVLEQLS FDKGFVFTKD VSYVQFLDRV REEERILRSI GMWDVPHPWL NLFVPQSRIL DFDTGVLKGV FVGAN-PVGV ILMYPMNRNM
OsCKX7  MYYN---EST ST----TMDQ KLESILGQLS FEEGFVFTKD VRYVQFLDRV REEERVLRSI GMWDVPHPWL NLFVPRSRIL DFDAGVFKGV FAGAN-PVGV ILMYPMNTNM
OsCKX8  VHDF---QQD SS----RADH VVKLVSAKLS YLRPHVYSVE VSYFDFLNRV RMEEESLRSR GLWDVPHPWL NVFVPKHGIT QFKGLLMDTV SADD--FEGP ILVYPLLTDK
OsCKX9  KNFN---PDE AD----VMEQ EVNTLLSQLR YMPSSLFHTD VTYIEFLDRV HSSEMKLRAK GMWEVPHPWL NIIIPRSMIH KFAKEVFGKI LKDSN--NGP ILLYPVNKSR
OsCKX10 MYYD---DNT AA----SVDQ KLDALLEELS FVRGFVFVRD ASYVEFLDRV GREEQNLRSA GAWDVPHPWL NLFVPRSRIL HFDAAVFKGI LRNAN-PVGL ILMYPMNKDM
OsCKX11 LYQR---GGG GDGGGDDMDK RVGEMMRQLK YVRGLEFAAG VGYVDFLSRV NRVEDEARRN GSWAAPHPWL NLFISSRDIA AFDRAVLNGM LADG--VDGP MLIYPMLKSK
PsCKX1  LHYR---KAA RS---SEVDT KVDRLLGGLR FVEGVKFEDD VKYVDFLLRV KRVEEDAKAK GIWDAPHPWL NMFVSKSDIG DFDREVFKKI LKHG--VGGP ILVYPLLRSK
RfCKX1  KYFT---P-- ------PRRP DDDALLSSLQ YDSCAEYNSD VDYGDFINRM ADQELDLRHT GEWFYPHPWA SLLIPADKIE QFIETTSSSL TDDLG-NSGL IMVYPIPTTP
PvCKX   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
```

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TrCKX1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX3  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX4  YALNFFRWDN NMSATIPD-D EDD---VFYA VGFLHSSG-F D-----NWKA FDAQNKEILQ FCNDAE---- --IKYKLYLP HYNTQEEWTN -HFGPKKWKT FLQRKYEFDP
TrCKX5  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX6  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TrCKX7  -------WNS KTSLVTP--- EED---VFYL VAFLSSAVPS S-TGANSLEN IQAQNKRILD FCTSAQ---- --LNVKQYLP HYSTQQEWQD -HFGSD-GRN WWKGKQPMTH
AtCKX1  -------WKK HTSLITP--- NED---IFYL VAFLPSAVPN S-SGKNDLEY LLKQNQRVMN FCAAAN---- --LNVKQYLP HYETQKEWKS -HFGKR-WET FAQRKQAYDP
AtCKX2  -------WDN RMSAMIPE-I DED---VIYI IGLLQSAT-P K-----DLPE VESVNEKIIR FCKDSG---- --IKIKQYLM HYTSKEDWIE -HFGSK-WDD FSKRKDLFDP
AtCKX3  -------WND RMSAAIP--- EED---VFYA VGFLRSAG-F D-----NWEA FDQENMEILK FCEDAN---- --MGVIQYLP YHSSQEGWVR -HFGPR-WNI FVERKYKYDP
AtCKX4  -------WNN RMSTMTP--- DED---VFYV IGLLQSAGGS Q-----NWQE LENLNDKVIQ FCENSG---- --IKIKEYLM HYTRKEDWVK -HFGPK-WDD FLRKKIMFDP
AtCKX5  -------WDE RSSAVTP--- DEE---VFYL VALLRSALTD GEE-TQKLEY LKDQNRRILE FCEQAK---- --INVKQYLP HHATQEEWVA -HFGDK-WDR FRSLKAEFDP
AtCKX6  -------WDN QTSAVTP--- EEE---VFYL VAILTSASPG S-AGKDGVEE ILRRNRRILE FSEEAG---- --IGLKQYLP HYTTREEWRS -HFGDK-WGE FVRRKSRYDP
AtCKX7  -------WDD RTSVVIPEE- ----GEIFYI VALLRFVPPC AKV--SSVEK MVAQNQEIVH WCVKNG---- --IDYKLYLP HYKSQEEWIR -HFGNR-WSR FVDRKAMFDP
OsCKX1  -------WDD RMTAVSG--- NDD---MFYV VGLLRSAVVP G-----DVER LERENEAVLA FCDNEG---- --IGCKQYLP HYASQDGWRS -HFGA-KWSR VTELKVKYDP
OsCKX2  -------WDS NMSAVIT--- DDDGDEVFYT VGILRSAAAA G-----DVGR LEEQNDEILG FCEVAG---- --IAYKQYLP YYGSQAEWQK RHFGANLWPR FVQRKSKYDP
OsCKX3  -------WDT NTSVVLPDSG STDQ--VMYA VGILRSANPD DGCSHHCLQE LLLRHRRLAG AAAS------ -GLGAKQYLA HHPTPAGWRR -HFGRR-WER FADRKARFDP
OsCKX4  -------WDN RTSVVIP--- DEE---IFYL VGFLSSAPS- S-SGHGSVEH AMNLNNKIVD FCEKNG---- --VGMKQYLA PYTTQKQWKA -HFGAR-WET FERRKHTYDP
OsCKX5  -------WDP RSSVVTP--- EED---VFYL VAFLRSAVPG STDPAQSLEA LERQNREILE FCDEAG---- --IGAKQYLP NHKAQREWEA -HFGAR-WAR FARLKAEFDP
OsCKX6  -------WDD RMTAVSG--- NDD---MFYV VGLLRSAVVP G-----DVER LERENEAVLA FCDNEG---- --IGCKQYLP HYASQDGWRS -HFGA-KWSR VTELKVKYDP
OsCKX7  -------WDD CMMAVAS--- DDD---VFYA VGLLRSAAVI G-----DVER LEKENEAVLA FCHNED---- --IGCKQYLP YYTSQDGWQR -HFGA-KWSR VADLKAKYDP
OsCKX8  -------WDG NTSAVVP--A APDG--VMYI FGVLRSTDPA R-CGRACVDS IMARHRRVAD EACRDGGGGG RGIGAKQYLA RQPSPARWRD -HFGAG-WGR FAARKARFDP
OsCKX9  -------WDN RTSVVIP--- DEE---VFYL VAFLSSA--- --LGPHNIKH TLDLNYRIIE FSDKAG---- --IGVKQYLP NYTTEQEWQS -HFGAR-WDT FQQRKKAYDP
OsCKX10 -------WDD RMTAMTP--- DED---VFYA VGLLRSAVAG GSG--GDVEQ LERENAAVLE LCDLAGG--- -GIGCRQYLP HHASRDGWRR -HFGA-KWGR VADLKARYDP
OsCKX11 -------WDP ATSVALPN-- ----GEIFYL VALLRFCRPY PGGG-PPVDE LVAQNNAIID ACRSNG---- --YDYKIYFP SYHAQSDWSR -HFGAK-WSR FVDRKARYDP
PsCKX1  -------WDD RHSVVVPD-- ----SNIFYI IALLRFIPPP PKG--PPTEK LVAQNNAIIQ LCYNKG---- --FNFKLYLP HYLSHENWMR -HFGDK-WNR FVQRKQNFDP
RfCKX1  --------IT APFIPIP--- HCD---TFFM LAVLRTASPG A------EAR MIASNRLLYE QARDVGG--- --VAYAVNAV P-MSPGDWCT -HFGSR-WQA IARAKRRFDP
PvCKX   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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TrCKX1  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
TrCKX2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
TrCKX3  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
TrCKX4  R--------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
TrCKX5  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
TrCKX6  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
TrCKX7  WHCLP----- ---------- ---------L AIGSFKR--- ---------Q CLHPVSLKLL G--------- ---------- ---------- ---------- ---------
AtCKX1  LAILAPGQRI FQKTTGKLSP I---QLAKSK ATGSPQRYHY ASILPKPRTV *KFPVSVLVT AQARQQESEK FFLFCFFW*- TTNFTV*--N RLGQLYRISI K--------
AtCKX2  KKLLSPGQDI F*-LT*CDMS F----VR--S NI*NYNIVT- --------I* **HFG-GSKI DQ*H------ ---------- ---------- ---------- ---------
AtCKX3  KMILSPGQNI FQKINSS*-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
AtCKX4  KRLLSPGQDI FN*LITCLN* F----VM*L* NVFL*HIQTS TFIV*LTNII V**FG*ATYV NLFRSCMKYI V*VKRQRKIY YYS------- ---------- ---------
AtCKX5  RHILATGQRI FQNPSLSLFP P----SSSSS SAASW*---- ---------- ---------- ---------- ---------- ---------- ---------- ---------
AtCKX6  LAILAPGHRI FQKAVSYS*P VVSWNYLGPL SLSVVNSISK DCTYCFR*CV SIYVVSQFRT QQGR*RRRRK NQYAPTKYSF IPPFAKVGL* *CGLIYSLNF LSGPIGPQV
AtCKX7  MAILSPGQKI FNRSL*PLIF L-------LF LLFLFLLLYS *VLWSLCNFF CIFLFSQEKN QLVFYKRWPT SHVQL----- ---------- ---------- ---------
OsCKX1  YGILSPGQRI FSSLTP---- ---------M ALVAM*---- ---------- ---------- ---------- ---------- ---------- ---------- ---------
OsCKX2  KAILSRGQGI FTSPLA*--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
OsCKX3  RCILGPGQGI FPRDSSSSNG ---------- AFASYS*--- ---------- ---------- ---------- ---------- ---------- ---------- ---------
OsCKX4  LAILAPGQRI FPKAS----- -------LPM SL*------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
OsCKX5  RAMLATGQGI FDSP--PLLA E----S*--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
OsCKX6  YGILSPGQRI FSSLTP---- ---------M ALVAM*---- ---------- ---------- ---------- ---------- ---------- ---------- ---------
OsCKX7  HRILSPGQRI FSSPAS---- ---------M VVVSM*---- ---------- ---------- ---------- ---------- ---------- ---------- ---------
OsCKX8  LHVLGPGQGI FPR--TDSAG ---------- SM*------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
OsCKX9  LAILAPGQRI FQKAS----- -------ASL PLPS*----- ---------- ---------- ---------- ---------- ---------- ---------- ---------
OsCKX10 RAILSPGQGI FPPPPPPSPP P----PAAGE PITAS*---- ---------- ---------- ---------- ---------- ---------- ---------- ---------
OsCKX11 LAILAPG--- ---------- ---------- ---------- ------QNIF ARTPSSVAAA AAVIV*---- ---------- ---------- ---------- ---------
PsCKX1  MAILAPG--- --HFLRPL-- ---------- ---------- -----MKQHL STTLLSKDA* ---------- ---------- ---------- ---------- ---------
RfCKX1  YRILAPGYRM SFD------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
PvCKX   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------
```

6.4.15 ACTIN

6.4.15.1 CTR0036061012-cF6_20040726

Genomic DNA sequence for ACT from the Pastoral Genomics database. Coding sequences are displayed in upper case.

>ACT CTR0036061012-cF6_20040726
ctttcgaagtgtttcggttttgttgactggatttatggtctgatctgtgtttttatgcgtgaatttccctgtttttgttttcttttttgtagtcatggttttttctttatagaacttcatttggaattttgaacttttgttat
aacctgattcatagtaaattgaagattttagtacttatttgtgatgagaaacttgagattattggtttcacattttatgacattttgaatacagttctattgttcggttttttgtttataggactatgtggctgctctactt
tctttctttgaaaacctaaaattatctaatctaagttcttgaagaagaaatttgaaaatgactgttatttcatttttatttttttgcttcaatgctattgataaatgataattgtgcttgttctatacaatgtgaactattg
ctgttttatgtagaaatggttagtatttgaagcaaaactaatcttccacatttgtaattggctgaattctgtcggtggtcgttttgactagttagtactgacaatcttattcctgcatactgtagttcagtaaaagATGGC
TGATGCTGAGGATATTCAACCCCTTGTTTGTGACAATGGAACCGGAATGGTGAAGgttagtacttagtagtactttgaatatttttttttgtgatttttgtcttttattcttcttttttgttactaacaacttgtcaatctgt
aatctatgtgcagGCGGGTTTCGCTGGTGATGATGCTCCCAGGGCTGTGTTTCCAAGTATTGTTGGCCGACCACGTCATACCGGTGTTATGGTTGGGATGGGTCAAAAGGATGCCTATGTTGGTGATGAAGCCCAATCAAA
AAGAGGTATTCTTACTCTCAAGTACCCCATTGAGCATGGTATTGTCAGTAACTGGGATGACATGGAAAAGATCTGGCATCACACATTTTACAATGAATTGCGTGTTGCTCCTGAGGAGCACCCAGTGCTTCTAACTGAGGC
TCCACTCAACCCAAAGGCCAACAGAGAAAAGATGACCCAAATCATGTTCGAGACCTTTAATGTGCCTGCCATGTATGTGGCCATTCAGGCTGTCCTCTCCCTCTATGCAAGTGGTCGTACAACTggttcgtattttatcta
tctctcttagaacatacttttgaatctgatattccattattctgttactcaataaatcatatttgaaacaGGTATTGTCTTGGATTCTGGTGATGGTGTGAGTCACACTGTGCCAATCTACGAGGGTTATGCACTCCCCCA
TGCCATTCTTCGTTTGGATCTTGCTGGCCGTGATCTAACTGAGTCTTTGATGAAGATCCTCACTGAGAGAGGGTACATGTTCACCACCTCAGCTGAGCGGTAAATTGTTCGTGACATCAAGGAGAAGCTTGCCTATGTTGC
TGTGGATTATGAACAAGAACTTGAGACTGCAAAGAGCAGTTCTTCAATTGAGAAAAACTATGAGCTTCCTGATGGACAGGTTATCACAATTGGAGCTGAGAGGTTCCGTTGCCCTGAAGTTCTTTTCCAGCCATCTATGAT
TGGAATGGAAGCTGCTGGAATTCATGAGACCACCTACAACTCTATAATGAAGTGTGACGTGGATATCAGAAAGGATCTGTACGGAAACATTGTTCTCAGTGGTGGTTCAACTATGTTTCCTGGTATTGCTGACCGTATGAG
CAAGGAGATCACTGCCCTGGCTCCTAGCAGCATGAAGATTAAGGTTGTGGCTCCACCAGAGAGAAAGTACAGTGTCTGGATTGGAGGATCAATCCTTGCATCCCTCAGCACCTTCCAGcaggtgattattttatcacaaat
tagcacattgccatgtaatgccaattaaattgatttctaaatcatgtttttgtcttgCAGATGTGGATATCCAAGGGTGAATATGATGAGTCTGGCCCATCCATCGTCCATAGAAAGTGCTTCtaagtccaaaatgatgca
ataatggtaagttatttttgctgtggttgctttttggtgtctctgtcatgtcatatgaactatgttggtggataagaggtgagataggatcattggagggtatattctgagattgatttatc

6.4.15.2TrACT alignment of sequencing results

```
TrACT_CDS   ATGGCTGATGCTGAGGATATTCAACCCCTTGTTTGTGACAATGGAACCGGAATGGTGAAGGCGGGTTTCGCTGGTGATGATGCTCCCAGGGCTGTGTTTCCAAGTATTGTT
TrACT_F2    ------------------------------------------------------------------------------------------------------------
TrACT_R2    ------------------------------------------------------------------------------------------------------------
ACTN1_ActF1 ------------------------------------------------------------------------------------------------------------
ACTN1_ActR1 ------------------------------------------------------------------------------------------------------------

TrACT_CDS   GGCCGACCACGTCATACCGGTGTTATGGTTGGGATGGGTCAAAAGGATGCCTATGTTGGTGATGAAGCCCAATCAAAAAGAGGTATTCTTACTCTCAAGTACCCCATTGAG
TrACT_F2    ------------------------------------------------------------------------------------------------------------
TrACT_R2    ------------------------------------------------------------------------------------------------------------
ACTN1_ActF1 ------------------------------------------------------------------------------------------------------------
ACTN1_ActR1 ------------------------------------------------------------------------------------------------------------

TrACT_CDS   CATGGTATTGTCAGTAACTGGGATGACATGGAAAAGATCTGGCATCACACATTTTACAATGAATTGCGTGTTGCTCCTGAGGAGCACCCAGTGCTTCTAACTGAGGCTCCA
TrACT_F2    ----------------------------ATGGARAAGATYTGGCATCACA------------------------------------------------------------
TrACT_R2    ------------------------------------------------------------------------------------------------------------
ACTN1_ActF1 ----------------------------------------------------CATTTTACA-TGAGYTKCGTGTTGCTCCTGAGGAGCACCCMGTGCTTCTWACTGAGGCTCCW
ACTN1_ActR1 --------------------------YTATGGAGAAGATC-KGCATCACACATTTTACAATGARTTKCGTGTTGCTCCTGARGAGCACCCMGTGCTTCTAACTGAGGCTCCA

TrACT_CDS   CTCAACCCAAAGGCCAACAGAGAAAAGATGACCCAAATCATGTTCGAGACCTTTAATGTGCCTGCCATGTATGTGGCCATTCAGGCTGTCCTCTCCCTCTATGCAAGTGGT
TrACT_F2    ------------------------------------------------------------------------------------------------------------
TrACT_R2    ------------------------------------------------------------------------------------------------------------
ACTN1_ActF1 CTCAAYCCMAAGGCCAACAGAGAAAAGATGACCCAGATCATGTTYGAGACCTTTAATGTGCCTGCCATGTATGTKGCCATTCAGGCTGTCCTCTCCCTCTATGCWAGTGGW
ACTN1_ActR1 CTCAAYCCMAAGGCCAACAGAGAAAAGATGACCCAAATCATGTTYGAGACCTTTAATGTGCCTGCCATGTATGTGGCCATTCAGGCTGTCCTSTCYCTSTATGCWAGKG-T

TrACT_CDS   CGTACAACTGGTATTGTCTTGGATTCTGGTGATGGTGTGAGTCACACTGTGCCAATCTACGAGGGTTATGCACTCCCCCATGCCATTCTTCGTTTGGATCTTGCTGGCCGT
TrACT_F2    ------------------------------------------------------------------------------------------------------------
TrACT_R2    ------------------------TCTGGTGATGGTGTSWSYCAY--------------------------------------------------------------
ACTN1_ActF1 CGTACWACTGGTATTGTCTTGGATTCTGGTGTGGATGTCTCYCAT--------------------------------------------------------------
ACTN1_ActR1 CGTACAACAGGTCTTGCGTT----AGACCACTACCACASWSRGTR--------------------------------------------------------------

TrACT_CDS   GATCTAACTGAGTCTTTGATGAAGATCCTCACTGAGAGAGGGTACATGTTCACCACCTCAGCTGAGCGGTAAATTGTTCGTGACATCAAGGAGAAGCTTGCCTATGTTGCT
TrACT_F2    ------------------------------------------------------------------------------------------------------------
TrACT_R2    ------------------------------------------------------------------------------------------------------------
ACTN1_ActF1 ------------------------------------------------------------------------------------------------------------
ACTN1_ActR1 ------------------------------------------------------------------------------------------------------------

TrACT_CDS   GTGGATTATGAACAAGAACTTGAGACTGCAAAGAGCAGTTCTTCAATTGAGAAAAACTATGAGCTTCCTGATGGACAGGTTATCACAATTGGAGCTGAGAGGTTCCGTTGC
TrACT_F2    ------------------------------------------------------------------------------------------------------------
TrACT_R2    ------------------------------------------------------------------------------------------------------------
ACTN1_ActF1 ------------------------------------------------------------------------------------------------------------
ACTN1_ActR1 ------------------------------------------------------------------------------------------------------------
```

6.4.16 GAPDH

6.4.16.1 CTR0036085039-cF6_20040726

Partial genomic DNA sequence for GAP. Data from the pastoral genomics database. Coding sequences are displayed in upper case.

```
>TrGAP CTR0036085039-cF6_20040726
tgttgttaatctatgatctgttgttttggatctgaatatctgttaattaataattaattagataattaattaatatgattgcatctgaataataggaaccctgaggagattccatggggtgaggttggagctgattatgtt
gttgagtcaactggtgttttcactgacaaggataaagctgctgctcatttgaaggttcatttcattaatctcttattattcttaattatttatttgattgattaattattactatgtatttgatgtggatcgtgtattttg
gttacaataccaaaaaagtcaaaaactatgatatgttttgtttgtgtcaactgttgttgacttgttttactgatagggtcaactagtatgttatgttttctttcttttgattgatttcgtagtgagtactaatgatgtat
tatgaatctagtaattaattagatttcaatgatttgaaatgtgtgtaatttggattgttgttatgattagGGTGGTGCTAAGAAGGTTGTTATTTCTGCTCCAAGCAAAGATGCACCTATGTTTGTTGTTGGTGTTAATGA
GAAGGAATACACATCAGATCTTAACATTGTTTCCAATGCTAGTTGCACTACCAATTGTCTTGCTCCCCTTGCCAAGGTTATTAACGATCGATTTGGAATCGTTGAGGGTCTTATGACCACTGTCCACGCCATCACAggttg
cttacttacacatttattcagtttaacctctattacacaggctatatttttttttgaattgttacatgatatttttttttttctatttgagataaatttgatatttattaacggtttattacgatatttttttttgtggtgaat
ctcaGCTACTCAGAAGACTGTTGATGGTCCATCAAGCAAGGACTGGAGAGGTGGAAGAGCTGCTTCCTTCAACATCATTCCCAGCAGCACTGGAGCTGCCAAGgtactctataaatctataatgatatgatataaatatgt
caaattatgttacatggttggatggtatacattttatttcaatttctatagcattgttaatgaatgttgaatttggataatgcagGCTGTAGGAAAGGTTCTTCCAGTATTGAACGGTAAATTGACCGGAATGTCTTTCCG
TGTCCCTACTGTTGATGTTTCAGTTGTTGACCTTACTGTAAGGCTTGAGAAGAAAGCAACCTATGATCAGATCAAAGCTGCTATCAAGtaaagtttaaaacttgaagcctttacattagaccaattgaaattattttccct
cctaacataacccattttgttgaaatgtcaaaaaccaggGAGGAATCAGAGGGCAAGCTCAAGGG
```

6.4.16.2TrGAP alignment with sequencing results and primers.

```
Tr_GAP_cds  GGTGGTGCTAAGAAGGTTGTTATTTCTGCTCCAAGCAAAGATGCACCTATGTTTGTTGTTGGTGTTAATGAGAAGGAATACACATCAGATCTTAACATTGTTTCCAATGCT
TrGAP_F2    ------------------------------------------------------------------------------------------------------------
TrGAP_R2    ------------------------------------------------------------------------------------------------------------
GAPN1_GpaF2 ------------------------------------------------------------------------------------------------------------
GAPN1_GpaR2 ------------------------------------------------------------------------------------------------------------

Tr_GAP_cds  AGTTGCACTACCAATTGTCTTGCTCCCCTTGCCAAGGTTATTAACGATCGATTTGGAATCGTTGAGGGTCTTATGACCACTGTCCACGCCATCACAGCTACTCAGAAGACT
TrGAP_F2    ----------------------------------------------------GGAATCGTTGAGGGTCTTATGA----------------------------------
TrGAP_R2    ------------------------------------------------------------------------------------------------------------
GAPN1_GpaF2 ------------------------------------------------------------------------GCACATGTYMYGTCMTCAMTGCTMCTCAGAAGACT
GAPN1_GpaR2 ----------------------------------------------------TTRGGAATCGTTGGAGGTCTTATGACCACTGTYCAYKCCATCACAGCYACTCAGAAGACT

Tr_GAP_cds  GTTGATGGTCCATCAAGCAAGGACTGGAGAGGTGGAAGAGCTGCTTCCTTCAACATCATTCCCAGCAGCACTGGAGCTGCCAAGGCTGTAGGAAAGGTTCTTCCAGTATTG
TrGAP_F2    ------------------------------------------------------------------------------------------------------------
TrGAP_R2    ------------------------------------------------------------------------------------------------------------
GAPN1_GpaF2 GTTGATGGTCCATCAASCAARGACTGGAGAGGTGGAAGAGCYGCYTCCTTCAACATYATTCCYAGCAGCACTGGAGCTGCYAAGGCTGTWGGAAARGTTCTTCCWGYWTTG
GAPN1_GpaR2 GTTGATGGTCCATCAAGCAAGGACTGGAGAGGTGGAAGAGCTGCTTCMTTCAACATCATTCCCAGCAGCACTGGAGCTGCCAAGGCTGTAGGAAAGGTTCTTCCAGTATTG

Tr_GAP_cds  AACGGTAAATTGACCGGAATGTCTTTCCGTGTCCCTACTGTTGATGTTTCAGTTGTTGACCTTACTGTAAGGCTTGAGAAGAAAGCAACCTATGATCAGATCAAAGCTGCT
TrGAP_F2    ------------------------------------------------------------------------------------------------------------
TrGAP_R2    ------------------------------------------------------------------------------------------------------------ACGA
GAPN1_GpaF2 AAYGGWAAATTGACCGGAATGKCWTTCCGTGTCCCWACTGTTGATGTYTCAGTTGTTGACCTTACTGTRAGGCTYGAGAARAMWGCAACCTATGATCARATYAAAGCTGCT
GAPN1_GpaR2 AACGGTAAATTGACCGGAATGTCTTTCCGTGTCCCTACTGTTGATGTTTCAGTTGTTGACCTTACTGTAAGGC-CGAGAAGAAAGCAACCTAK-ATCAGATCAAAGCTT--

Tr_GAP_cds  ATCAAGGAGGAATCAGAGGGCAAGCTCAAGGG
TrGAP_F2    --------------------------------
TrGAP_R2    TAGTTCCTCCTYAGTCTCC-------------
GAPN1_GpaF2 ATCAAGRGTGAATCTGAGGA------------
GAPN1_GpaR2 --------------------------------
```

6.4.17 PP2 alignment showing the PCR primers and sequencing results.

```
M_sativa_PP2                    ATGGTTGCAAATCAATTGTATGAGCTCTGTGAAGCTGTAGGCCCCGAACCTACCAGAGCGGAATTGGTCCCTGCATATGTTCGATTGCTTCGAGATAAT
P.sativum_PP2                   ATGGTTGCCAATCAACTCTATGAGCTCTGTGAAGCTGTTGGTCCTGATTCCACCAAGACGGAATTGGTTCCTGCATATGTTCGGCTGCTGCGTGATAAT
ETRN16RX12E03-g1M13RE_20030618 ATGGTTGCCAATCAACTCTATGAGCTCTGCGAAGCTGTTGGTCCTGATTCCACCAAGACGGAATTGGTTCCTGAAGATGTTCGGCTGCTGCGTGATAAT
CTR0036078442-cF2_20040726      ------------------------------------------------------AGGACGGAATTGGTTCCTGCTTATGTTCGGCTGCTGCGTGATAAT
ETOSTORX15A07-g1M13RE           ---------------------------------------------------------------------------------------------------
Primers_TrPP2_F2_Rr2            ----------------------------------------------------------------------------TvCCTGAAGATGTTCGGCTG------------
PP2_F2                          ---------------------------------------------------------------------------------------ATGCGTGATAT-
PP2_R2                          ------------------------------------------------------------------TSCCTGAAGATGTTCGGCTGCTGCGTGATAAT

M_sativa_PP2                    GAGGCTGAGGTACGCATAGCAGCTGCTGGGAAAGTGACCAAGTTTTGTCGGATTTTAAGTCCAGATCTTGCGATTCAGCATATTCTTCCTTGTGTGAAG
P.sativum_PP2                   GTGGCGGAAGTACGTATTGCTGCTGCTGGGAAAGTGTCTAAGTTCTCCCGCATATTAAGTCCTGAACTAGCCATTCAGCATATTCTACCATGTGTAAAG
ETRN16RX12E03-g1M13RE_20030618 GAAGCTGAAGT---------------------------------------------------------------------------------------
CTR0036078442-cF2_20040726      GAAGCTGAAGTACGTATTGCTGCTGCTGGGAAAGTGACTAAGTTCTCCCGCATATTAAGTCCTGAACTAGCCATTCAGCATATTCTACCATGTGTAAAG
ETOSTORX15A07-g1M13RE           ---------------ATTGCTGCTGCTGGGAAAGTGACCTTTTTCTCCCGCATATTAAGTCCTGAACTAGCCATTCAGCATATTCTACCATGTGTAAAG
Primers_TrPP2_F2_Rr2            ---------------------------------------------------------------------------------------------------
PP2_F2                          -GAGCTGA-GTACGTATTGCTGCTGCTGGGAA-GTGACTAAGTTCTCCCGCATATTAAGTCCTGAACTAGCCATTCAGCATATTCTACCAWGTGTAAAG
PP2_R2                          GAAGCTGAAGTACGTATTGCTGCTGCTGGGAAAGTGACTAAGTTCTCCCGCATATTAAGTCCTGAACTAGCCATTCAGCATATTCTACCATGTGTAAAG

M_sativa_PP2                    ------GAATTGTCATCAGACTCTTCACAGCATGTCCGTTCTGCACTGGCTTCAGTAATAATGGGAATGGCTCCTGTGTTAGGAAAGGAAGCAACAATA
P.sativum_PP2                   ------GAGTTATCAACAGATTCATCTCAACATGTTCGCTCCGCACTGGCTTCGGTTATAATGGGAATGGCACCGGTCTTAGGGAAGGATGCAACAATT
ETRN16RX12E03-g1M13RE_20030618 ---------------------------------------------------------------------------------------------------
CTR0036078442-cF2_20040726      ATACAGGAATTATCGACAGATTCATCACAACATGTTCGCTCTGCATTGGCTTCAGTTATAATGGGAATGGCACCAGTCTTGGGGAAGGATGCAACAATT
ETOSTORX15A07-g1M13RE           ------GAATTATCGACAGATTCATCTCAACATGTTCGCTCTGCATTGGCTTCAGTTATAATGGGAATGGCACCAGTCTTGGGGAAGGATGCGACAATT
Primers_TrPP2_F2_Rr2            ----------------------------------------------------GTCAWTATTACCCTTACCGTGG-------------------------
PP2_F2                          ------GAATTATCGACAGATTCATCTCAACATGTTCGCTCTGCATTGGCTTCAGTWATAWGGRGGAAWGGCACCW----------------------
PP2_R2                          ------GAATTATCGACAGATTCATCTCAACATGT-CGCTCTGCAT--GCT-----------------------------------------------

M_sativa_PP2                    GAGCAGCTTCTTCATATTTTCCTA--
P.sativum_PP2                   GAGCAACTTCTCCCTATTTTCCTCGC
ETRN16RX12E03-g1M13RE_20030618 -------------------------
CTR0036078442-cF2_20040726      GAGCAGCTTCTCCCGATTTTCCTC--
ETOSTORX15A07-g1M13RE           GAGCAACTTCTCCCGATTTTCCTC--
Primers_TrPP2_F2_Rr2            -------------------------
PP2_F2                          -------------------------
PP2_R2                          -------------------------
```

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